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pH-dependent structural reorganization of phosphatidylcholine vesicle membranes by copolymers of 2-ethylacrylic acid and methacrylic acid/

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pH-DEPENDENT STRUCTURAL REORGANIZATION OF PHOSPHATIDYLCHOLINE VESICLE MEMBRANES BY COPOLYMERS OF 2-ETHYLACRYLIC ACID AND METHACRYLIC ACID

A Dissertation Presented

by

HONG YOU

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This dissertation is dedicated to my mom,

my wife, Ockjin and my son, Bin.

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ABSTRACT

pH-DEPENDENT STRUCTURAL REORGANIZATION OF
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Copolymers of 2-ethylacrylic acid (EAA) and methacrylic acid (MAA) were prepared in bulk and in N,N-dimethylformamide (DMF). Best-fit terminal-model reactivity ratios were determined by a non-linear least squares technique to be r_{MAA} = 1.14 and r_{EAA} = 0.23 in bulk, and r_{MAA} = 1.91 and r_{EAA} = 0.09 in 50 % DMF solution, respectively. Examination of ^{13}C NMR spectra provided convincing evidence for the formation of statistical copolymers.

The pH-dependent structural reorganization of dipalmitoylphosphatidylcholine vesicle membranes could be effected in aqueous phosphate buffer solutions of EAA-MAA copolymers. The reorganization process is sensitive to the composition of the copolymer.

Decreasing the 2-ethylacrylic acid content in copolymer shifted the 'critical' pH to lower values. Copolymers of 2-ethylacrylic acid content equal to or less than 40 mole % showed complicated aggregation in the interaction with dipalmitoyl phosphatidylcholine vesicle membranes. Results from potentiometric titrations suggested that the shifts in the critical pH in the structural reorganization of DPPC vesicle membranes should be attributed to the different hydrophobic interactions of polymers.

Photosensitive 3,3'-dicarboxydiphenyliodonium bisulfate and hexafluorophosphate, which produced the strong acids (H_2SO_4, HPF_6) upon irradiation at 254 nm were prepared. Using these iodonium salts as proton sources, the structural reorganizations of phosphatidylcholine vesicle membranes by poly(2-ethylacrylic acid) were demonstrated by optical density measurement and by monitoring the efflux of the calcein in entrapped vesicles.

Copolymers of EAA and MAA were employed in specific enhancement of cytotoxicity of immunotoxins. It was found that the copolymer of 49 mole % 2-ethylacrylic acid content is not toxic to HeLa cells and potentiates the action of 5E7-gelonin immunotoxin. To improve the potentiating effect the copolymer was modified to have 0.9 and 1.6 mole % disulfide linkage in the side chain for attachment to the 5E7-gelonin molecule.

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CHAPTER I

INTRODUCTION

A. Phospholipid Vesicle Membranes

Phospholipids are complex molecules of biological origin, containing phosphorus in the polar head group, and a hydrocarbon region usually linked through glycerol or the sphingosine base, as in phosphatidylcholine or sphingomyelin, respectively. These molecules are ubiquitous to living organisms and are found in the membranes and membranous organelles of all living matter. Accordingly, in the attempt to understand the physical chemistry of biological membranes, an enormous number of research papers have been published on the study of model phospholipid bilayer membranes. Recently advances in the physiology and biochemistry of cell membranes (1) have been accompanied by increased interest in model membrane systems (2-4).

While most phospholipids have two aliphatic chains, as in phosphatidylcholine or sphingomyelin, the lyso derivatives have a single aliphatic chain and thus tend to be soluble amphiphiles. The two-chain molecules tend to be insoluble, swelling amphiphiles. Some phospholipids are even more complex in that two two-chain phospholipids are linked through the polar group to form a four-chain phospholipid, such as cardiolipin. The polar group may vary, from the zwitterionic phospholipids, such as phosphatidylcholine, to the charged lipids phosphatidylglycerol

and phosphatidic acid. The charge provides them with different physical properties at different states of ionization. Furthermore, charged moieties can readily interact with ionic moieties (e.g., metal ions) that invoke major changes in state. In addition, the hydrocarbon portion of the molecule may be saturated, such as in dipalmitoyl lecithin, or it may contain both saturated and unsaturated chains.

Liposomes are microscopic particles consisting of one lipid bilayer enclosing a single aqueous compartment (unilamellar vesicle, ULV) or a number of concentric bilayer enclosing an equal number of aqueous spaces (multilamellar vesicle, MLV). In the vesicle structure the long hydrocarbon chains form a hydrophobic core, while the hydrophilic headgroups contact the aqueous medium (see Figure 1.1). Dispersion of phospholipids in water with shaking or vortex agitation results in the formation of MLV's, which are relatively large (size range from 10^3 to 10^5 Å in diameter (5, 6) and consequently these preparations appear turbid due to scattering of incident light. Small unilamellar vesicles (SUV) of a more narrow size distribution can be prepared through sonication of MLV suspensions (7). The SUV's have diameters ranging from 200 to 500 Å (8).

The fundamental functions of biological membranes such as permeation and selectivity are closely associated with the gel-liquid crystal phase transition. Therefore, the phase transition would be one of the most essential functions provided by phospholipid biological membranes. Pure phospholipids undergo a reversible order-to-disorder transition with an increase in temperature through a characteristic melting temperature (T_m) (see Figure 1.2) (9). The transition has been associated with the order and alignment of the hydrocarbon chains within the bilayer structure

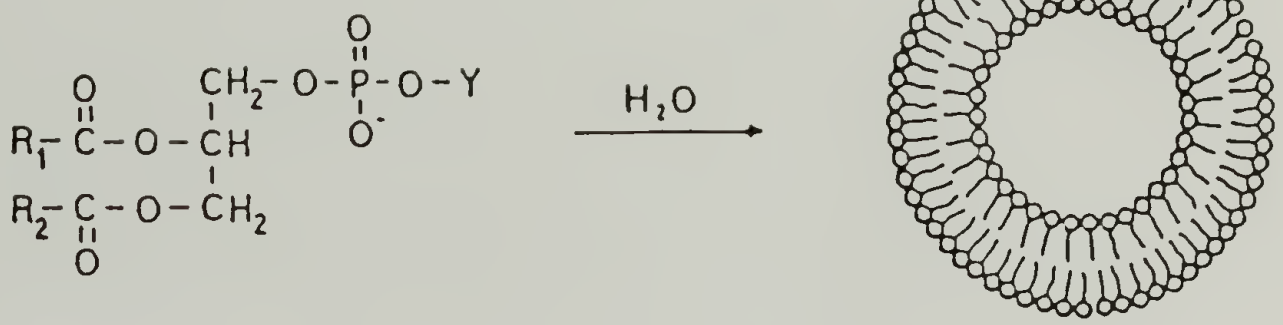


Figure 1.1 Formation of vesicular structures via the dispersion of phospholipid molecules in aqueous solution.

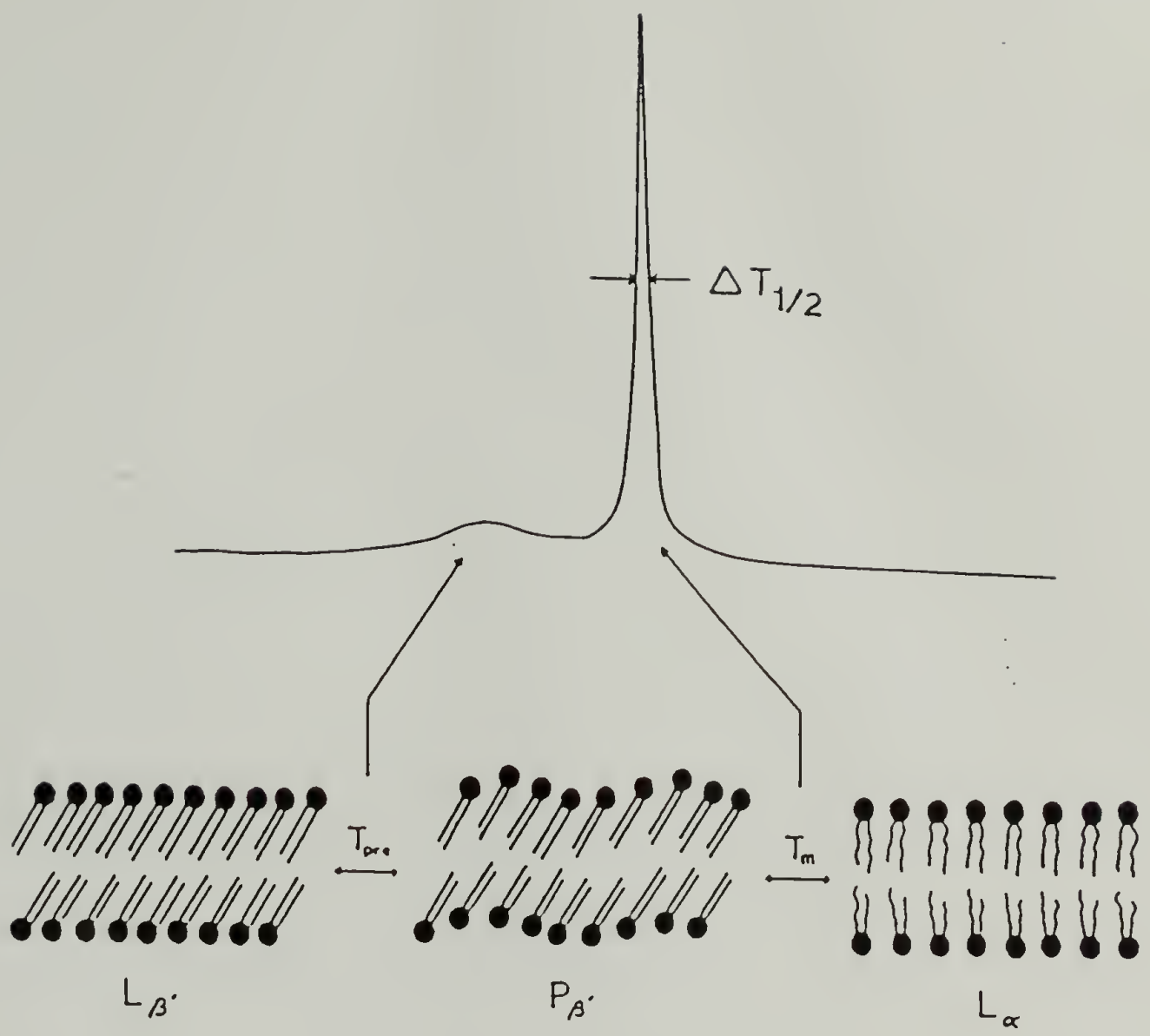
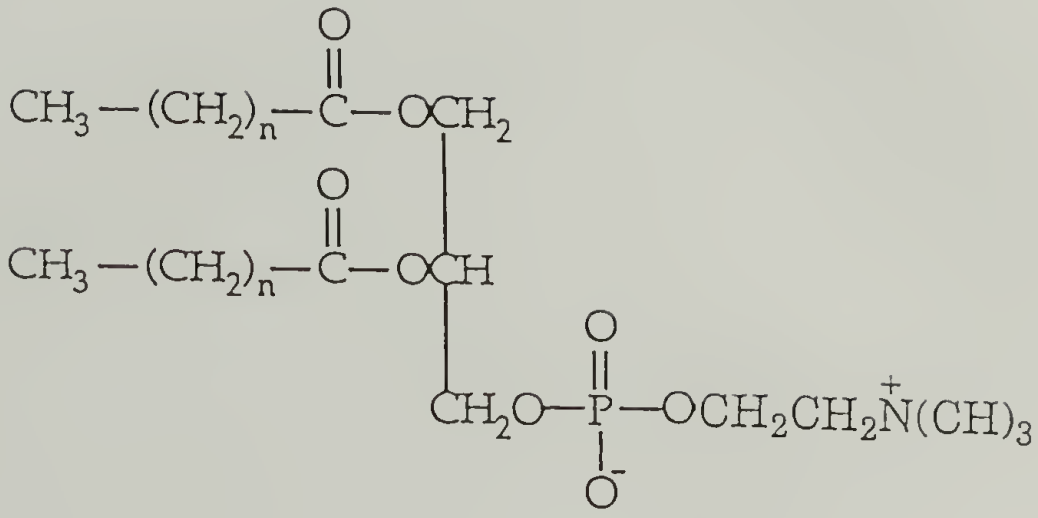


Figure 1.2 Phase transitions in phospholipid bilayers as detected by differential scanning calorimetry (DSC). The main melting transition (T_m) is associated with a change in the hydrocarbon chain conformation from ordered all-trans state ($L_{\beta'}$) to a disordered fluid-like state (L_α). Some exhibit a minor thermal transition known as a pertransition, which results in the formation of a periodic lamellar phase ($P_{\beta'}$). From Janiak, M. J., Small, D. M., and Shipley, G. G., Biochemistry, 15, 4574, 1976.

(10, 11). In the ordered gel state (denoted as $L_{\beta'}$) the hydrocarbon chains assume an all trans-configuration, while in the disordered fluid state (denoted as L_{α}) the chains contain several gauche rotamers. In multilamellar vesicles, the melting transition occurs over a very narrow temperature range (transition peak width at half-height, $\Delta T_{1/2}$, 0.5 °C), which is indicative of a highly cooperative process (9). Because the hydrocarbon chains are in close proximity, when one chain undergoes a trans to gauche rotation it induces a similar rotation in neighboring chains. The cooperative unit in phospholipid vesicles can include several hundred molecules. Some phospholipids exhibit a minor phase transition at temperatures below the main melting transition (see Figure 1.2). Known as the pre-transition, it is accompanied by a change to a periodic lamellar phase (denoted as $P_{\beta'}$) and leads to a rippled surface texture (11, 12). Liposomes formed from DPPC (see Figure 1.3) display a sharp transition at 41.4 °C (13). The main transition properties are shown for various PC in Table 1.1(13).

Following the demonstration by Bangham et al. that aqueous dispersions of phospholipids forms closed structures that are relatively impermeable to entrapped ions, there has been a great expansion in the use of lipid vesicles as model membrane systems and more recently as drug delivery systems. The main features of lipid vesicles that have made them a valuable investigative tool are the following ; (a) their characteristic morphology, where a relatively impermeable lipid bilayer completely encloses an aqueous space, and (b) their ability to encapsulate various solutes present in the aqueous phase during their formation. Among the applications of phopholipid bilayers and vesicles, one obvious use is as a



n=10, Dilauroyl phosphatidylcholine (DLPC)

n=12, Dimyristoyl phosphatidylcholine (DMPC)

n=14, Dipalmitoyl phosphatidylcholine (DPPC)

n=16, Distearoyl phosphatidylcholine (DSPC)

Figure 1.3 Chemical structure of some phosphatidylcholine lipid molecules.

Table 1.1 Melting transition temperatures of some saturated phosphatidylcholines.

Lipid	# of methylenes in R	T_m(°C)
DLPC	10	-1.8
DMPC	12	23.9
DPPC	14	41.4
DSPC	16	54.9

research tool for modelling those cellular functions which are determined at the membrane level. For example, greater understanding of the mechanism of action of pharmacologically active molecules may dictate that certain liposomes could be used as cell analogues, in predictive screens for certain classes of drugs.

The other applications are in the field of microanalysis and diagnostics. The studies by Kinsky (15-17) have shown that lipid-linked haptens can be incorporated into the phospholipid bilayer to form immunologically active liposomes. In the presence of complement, these liposomes undergo lysis when exposed to specific antibodies and, in the process, can release trapped marker substances. Hsia and Tan (18) have shown that this phenomenon forms the basis of a sensitive analytical method which they called membrane immunoassay.

The ability of the liposome to transfer normally non-penetrating molecules into cells and organelles could be particularly useful for transporting fragments of genetic material from cell to cell.

B. Poly(carboxylic acid)s in Aqueous Solution - Conformational Transition Behavior

Cohesive intramolecular interactions among apolar moieties have long been recognized as a major factor determining the unique compact conformations of globular proteins in aqueous solution. Such hydrophobic interactions strongly influence the solution dimensions of a number of synthetic water-soluble polymers as well.

Various synthetic polyelectrolytes with hydrophobic side chains have been known to show conformational transitions which are very similar to protein denaturation. The compact coil-expanded coil transition of polyelectrolytes with pH change has been investigated in detail for PMAA (19, 20, 44) by potentiometric titration or fluorescence spectrometry (see Figure 1.4). Copolymers of maleic acid with different alkyl vinyl ethers or with styrene (21-23) and copolymers of methacrylic acid with styrene have also been examined (24). The pK_a curves as a function of the degree of ionization of PMAA have a specific form ; a sharp initial rise, a plateau, and a slower rise in the region of the degree of ionization similar to the rise of the PAA curves (see Figure 1.5). The curves of this form are characteristic for molecules in which ionization is accompanied by a cooperative conformational transition that increases the mean distance between charged groups. In Figure 1.5, during the first part of the titration curve of PMAA, the electrostatic repulsion forces a cooperative expansion of the chain. Thus pK_a values stay as a plateau. When large expansions are reached, the influence of the conformational free energy will level off the further increase of the dimensions with increasing charge and the electrostatic potential will start to rise again (and pK_a also increases). The origin of the forces responsible for the stabilization of the compact state at low values of the degree of ionization is a hydrophobic interaction associated with the methyl side chains. From the titration behavior of partially esterified PMAA, Mandel and Stadhouder (25) concluded that the stabilization of the mean conformation at low values of degree of ionization is not due to hydrogen bonding between the carboxylic groups and should be ascribed to hydrophobic interactions between the

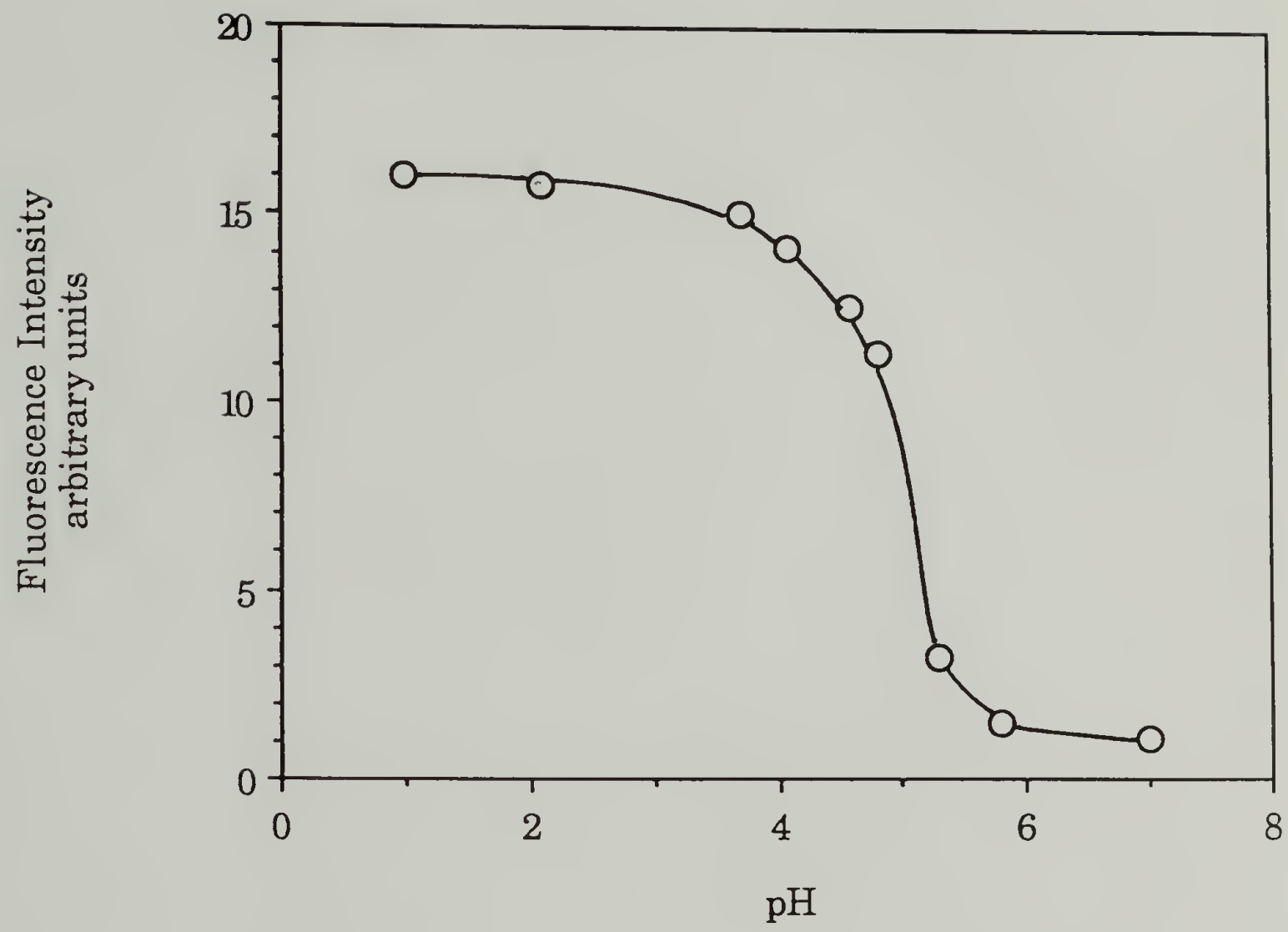


Figure 1.4 The conformational transition of the hydrophobic polyelectrolyte, PMAA monitored by using a hydrophobic fluorescent probe. The intensity emitted by 10^{-5} M pyrene in aqueous solutions of PMAA was measured at 373 nm with excitation wavelength of 337 nm. From Chen, T. and Thomas, J. K., J. Polym. Sci., Polym. Chem. Ed., 17, 1103, 1979.

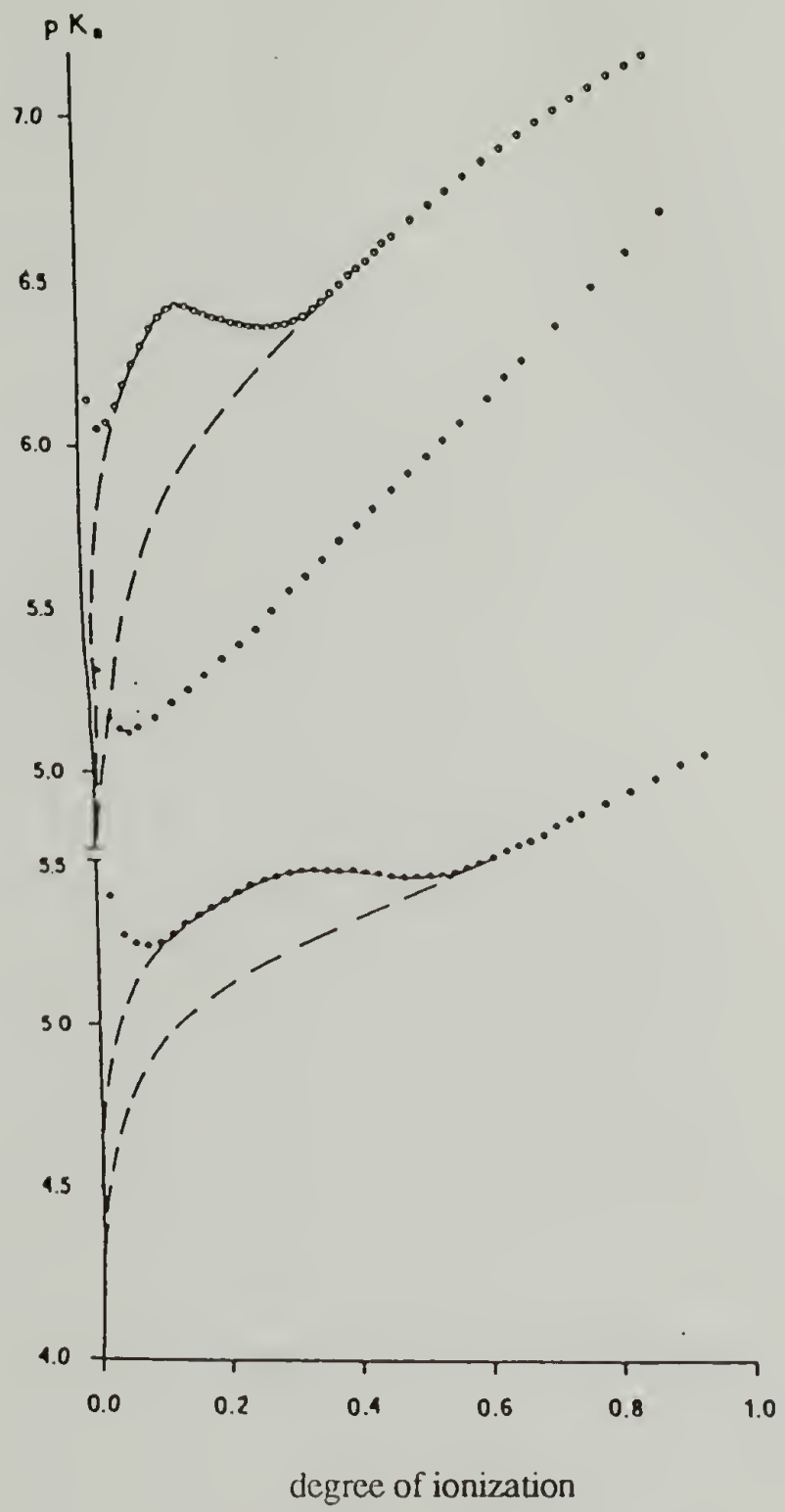


Figure 1.5 Potentiometric titration curves of PMAA and PAA, initial polymer concentration 4 x 10^{-2} equiv/l: (o) syndiotactic PMAA in water; (⊖) syndiotactic PMAA in 0.5 M NaCl; (●) PAA in water. Broken curves represent extrapolations based on Henderson-Hasselbach plots. From Crescenzi, V., Quadrifoglio, F., and Delben, F., J. Polym. Sci., Part A-2, 10, 357, 1972.

methyl side chains . The monotonous increase of pK_a with the increase of the degree of ionization found in PAA agrees with the theoretical curves of the titration of flexible polyelectrolytes without any structure (26, 27).

Compared to PAA which does not demonstrate such a pH-induced conformational transition, the presence of an extra methyl group in PMAA causes the polymer to unfold in a cooperative fashion with increasing ionization because of intramolecular hydrophobic interactions (19, 28-33). PEAA then exhibits a more cooperative unfolding with increasing the degree of ionization. The ionization at which the transition takes place is shifted to higher values for PEAA compared to PMAA due to stronger hydrophobic interactions in the former compound (34). The conformational transition of PEAA has been investigated by viscometric (35), ^1H NMR (35), optical (35), potentiometric (34, 36), fluorometric (37-39) (see Figure 1.6), and dynamic light scattering measurements (40, 41). Recently, Tirrell and coworkers investigated the effect of molecular weight of PEAA on the conformational transition behavior by potentiometric titration (42).

PMAA aqueous solutions with low values of degree of ionization display the ability to increase the solubility of some simple hydrocarbons (43, 44). This effect is correlated with tightly coiled conformations of PMAA at low values of the degree of ionization ($\alpha < 0.2$) in dilute aqueous solution and is attributed to the presence of hydrophobic domains in the interior of each tightly coiled polymer chain where the hydrocarbon molecules are preferentially located.

Tirrell and coworkers found that PEAA acts in an analogous manner to solubilize phospholipid molecules at low values of degree of ionization with the resultant formation of polymer-phospholipid mixed micelles (37).

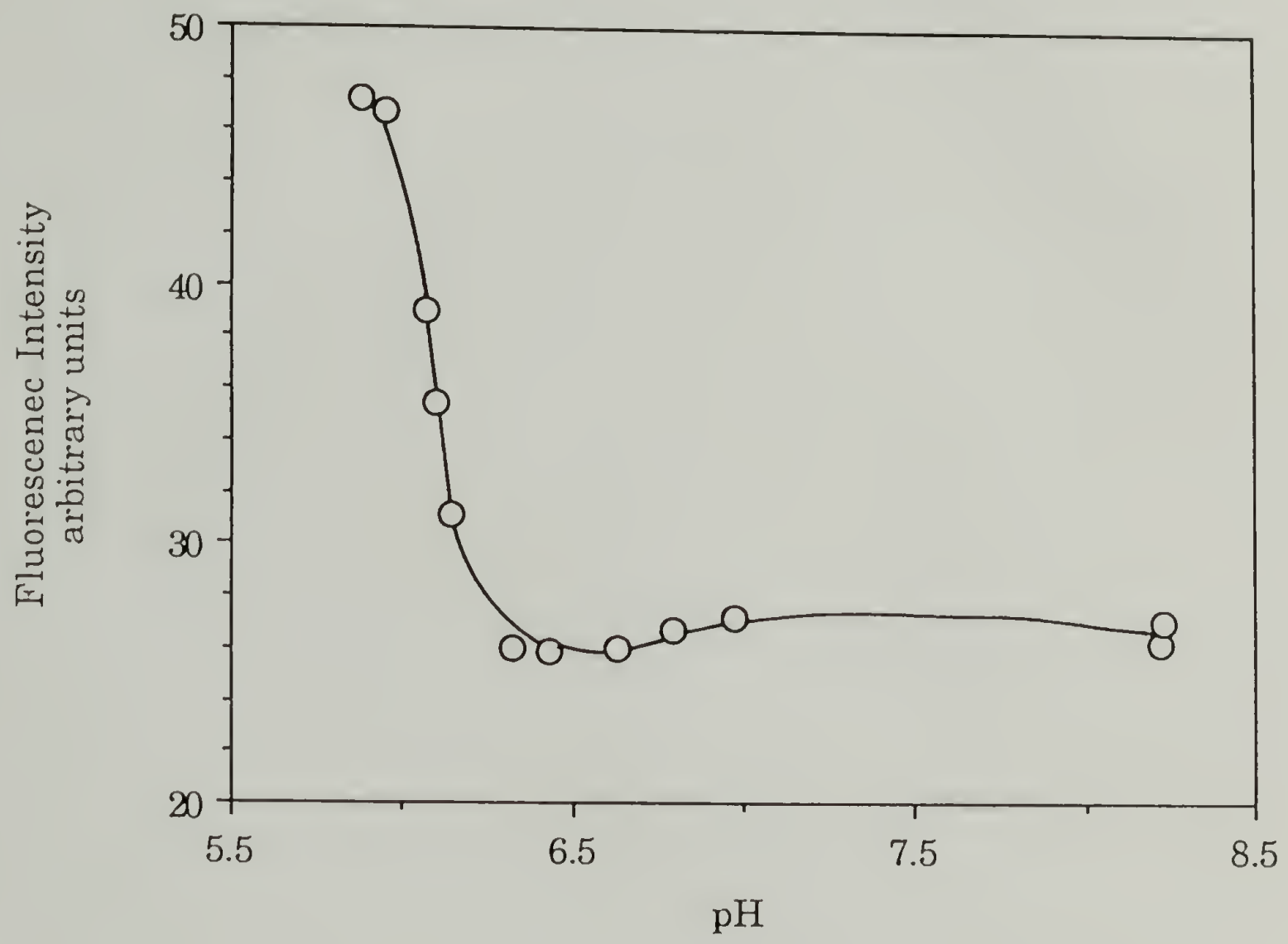


Figure 1.6 Fluorescence intensity at 374 nm emitted with excitation wavelength 337 nm by pyrene (5×10^{-6} M) in phosphate buffered solutions (0.02 M) of PEAA (1 mg/ml) as a function of pH at 23 °C. From Borden, K. A., Ph.D. Thesis, University of Massachusetts, 1989.

C. Interaction of Poly(carboxylic acid)s with Phospholipid Vesicle Membranes

Investigations of phospholipid membranes and synthetic polymers could be expected to play important roles in the pursuit of scientific knowledge of the structures and functions of biological membranes and macromolecules. These investigations are also of importance for the synthesis of novel functional polymers for reactions in organized media and to mimic the functions of biological molecules. The application of phospholipid bilayers and vesicles outside the biomedical sphere, in microelectronics, photoenergy conversion and in more selective catalysts, has also been reported (45).

Phospholipid vesicles have been being studied as vehicles for delivery of pharmaceutical agents (46-52). However, a major barrier to their use is the difficulty of directing them to specific target sites. More specific targeting has been attempted by use of a recognition macromolecule to bind liposomes selectively to particular cells, such as antibodies (53-55) and plant lectins (56). Different approaches, e.g., the use of local hyperthermia (57, 58) or locally acidic conditions (59) to promote selective delivery of liposome-encapsulated drugs to target areas have been tried. They suggested that pH-sensitive vesicles may prove useful in the selective delivery of drugs to targets of low ambient pH, such as inflamed or infected regions and certain tumor tissues. Local hyperthermia has received increased attention as a therapeutic tool, for use either alone or in conjunction with radiation (60) or drugs (61-63). Since many normal mammalian cells begin to show damage at about 42 ^{o}C the aim has been to

achieve the therapeutic results just a few degrees above physiological temperature. For both cases, proper design of liposomes for enhanced local release by hyperthermia or by locally acidic conditions was tried. For example, the preparation of pH-sensitive phosphatidylcholine vesicles was achieved by incorporating a small amount of N-palmitoyl-L-homocysteine (PHC) into the vesicle membranes (59).

Controlled release of vesicle-entrapped substances in response to changes in environmental conditions provides a basis of new technologies in pharmaceutics, in imaging, in sensing, or in medical diagnostics. Tirrell (64-68) and others (69-82) have demonstrated several means of preparing synthetic bilayer membranes that respond to signals. Tirrell and coworkers discovered that poly(2-ethylacrylic acid) (PEAA) undergoes a conformational transition to a globular structure upon acidification of its aqueous solutions (see Figure 1.7). Addition of the hydrophobic PEAA to aqueous dispersions of natural or synthetic phophatidylcholines, renders the lipid membranes exceedingly sensitive to pH (see Figure 1.8) (64). pH sensitivity arises from a cooperative collapse of the polyelectrolyte chain from an expanded, hydrophilic state in basic solutions to a globular, hydrophobic coil upon acidification (65). The globular polymer associates strongly with bilayer vesicles prepared from phosphatidylcholines and phosphatidylglycerols, and causes profound disruption of the bilayer organization. The overall change in aggregate morphology is accompanied by an abrupt change in solvation and conformation of polymer chain, but adsorption must precede conformational collapse (37). As chain ionization is depressed further, desolvation of the polymer causes the bilayer

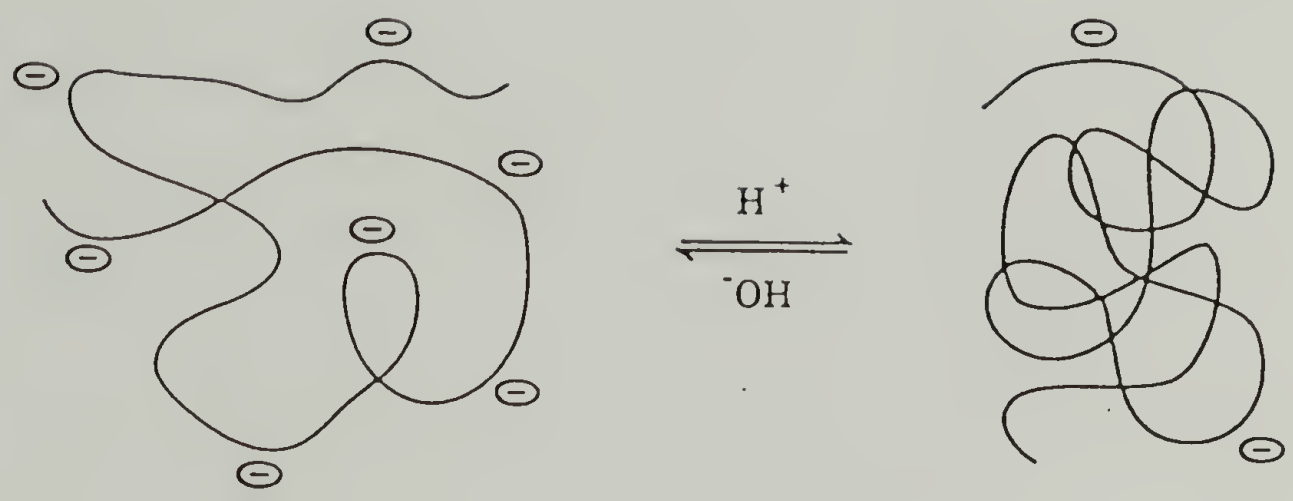


Figure 1.7 The pH-induced conformational transition of PEAA.

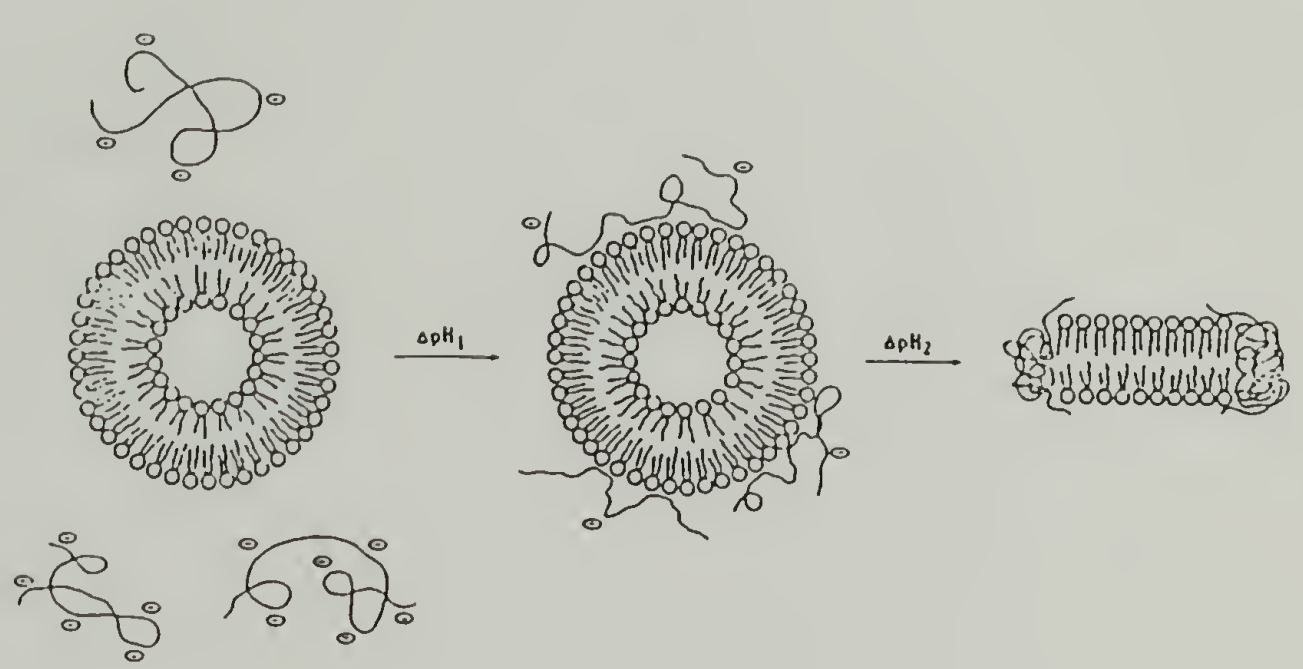


Figure 1.8 The mechanism of vesicle membrane reorganization by PEAA in aqueous solution. Conformational transition occurs in acidic solution with an increase of hydrophobicity of polyelectrolyte. Dehydration of the chain leads to reorganization of lipid bilayer from vesicular form at high pH to a mixed polymer-lipid micelle at low pH.

aggregate to become unstable with respect to a mixed micelle, in which water-polymer contacts are reduced (37).

Reorganization of DPPC vesicle membranes in aqueous solutions of PEAA is characterized by clarification of the highly turbid vesicle suspensions, which suggests the formation of small PEAA-DPPC mixed micelles. This phenomenon is shown in Figure 1.9 as a plot of optical density versus pH for mixtures of PEAA and DPPC. The optical densities of the mixtures of PEAA and DPPC vesicles decrease due to a reduction in size of the lipid containing particles. This was studied in detail by Borden et al. (37) by electron microscopy. Transmission electron micrographs of PEAA/DPPC systems were examined during the reorganization process, and revealed a transition of vesicles at high pH into a mixed polymer-lipid micelle having dimensions of 54 ± 6 Å in thickness and 160 ± 50 Å in diameter at low pH. Similar disk-like structures have been observed as the recombinant products from the interaction of phospholipid vesicles with apolipoproteins, bite salts, detergents, and amphiphilic proteins (83-89). They have also exploited these phenomena to prepare phospholipid vesicles that release their contents rapidly and quatitatively in response to small changes in pH (64), temperature (66), glucose concentration (67) or light intensity (68). Figure 1.10 shows the pH-dependent interaction of PEAA with phospholipid vesicles resulting in the release of contents entrapped in the internal aqueous cavity. The interaction of PAA and PMAA with DPPC vesicle membranes (64), was also investigated; however the same catastropic structural reorganization demonstrated with PEAA was not observed although pH-dependent complexation of PAA and PMAA with vesicle membranes was exhibited.

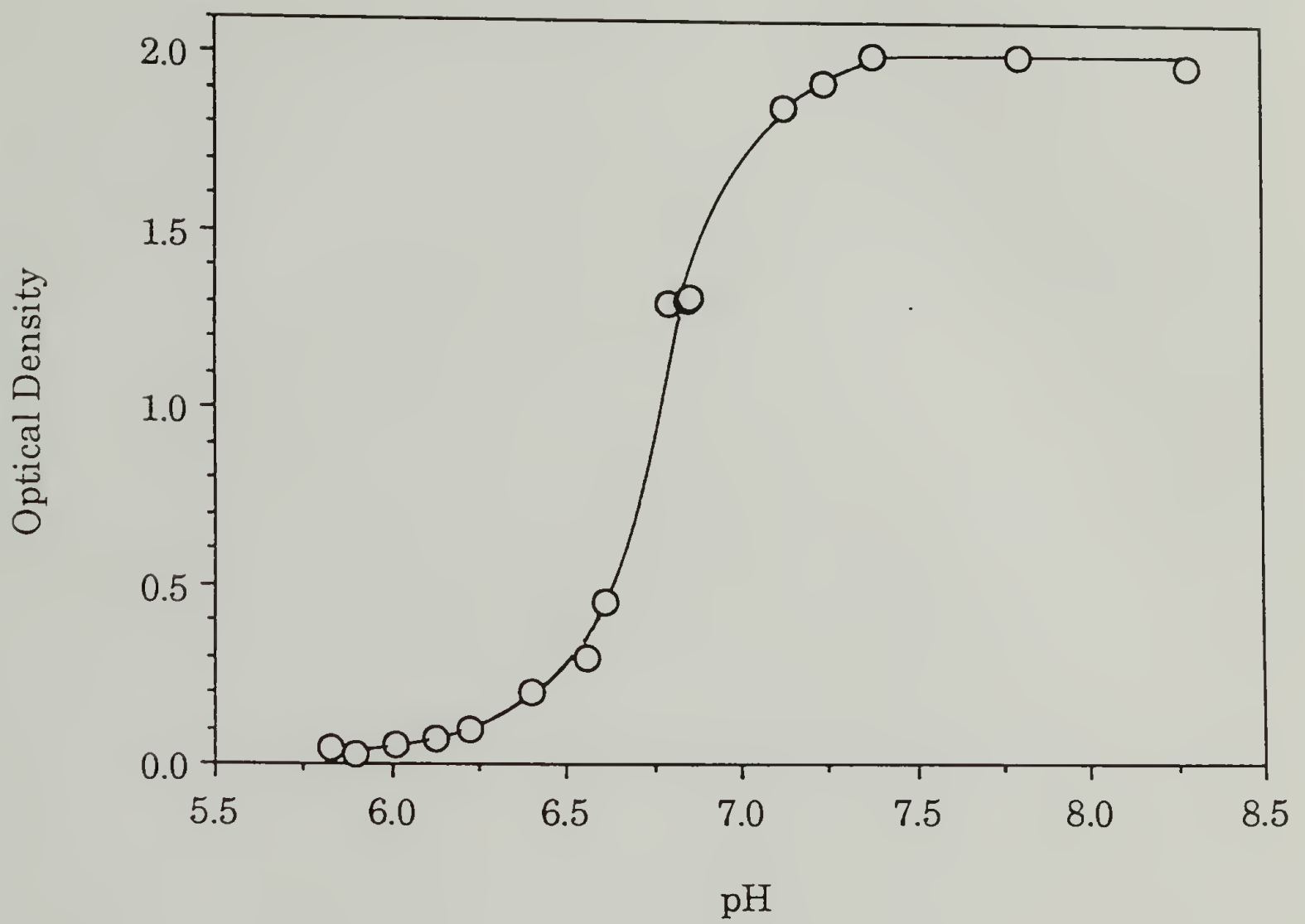


Figure 1.9 Optical density as a function of pH. Detection of the PEAA-induced reorganization of DPPC vesicles (MLV); DPPC and PEAA (1 mg/ml each) in 0.02 M phosphate buffer. From Borden, K. A., Ph.D., Thesis, University of Massachusetts, 1989.

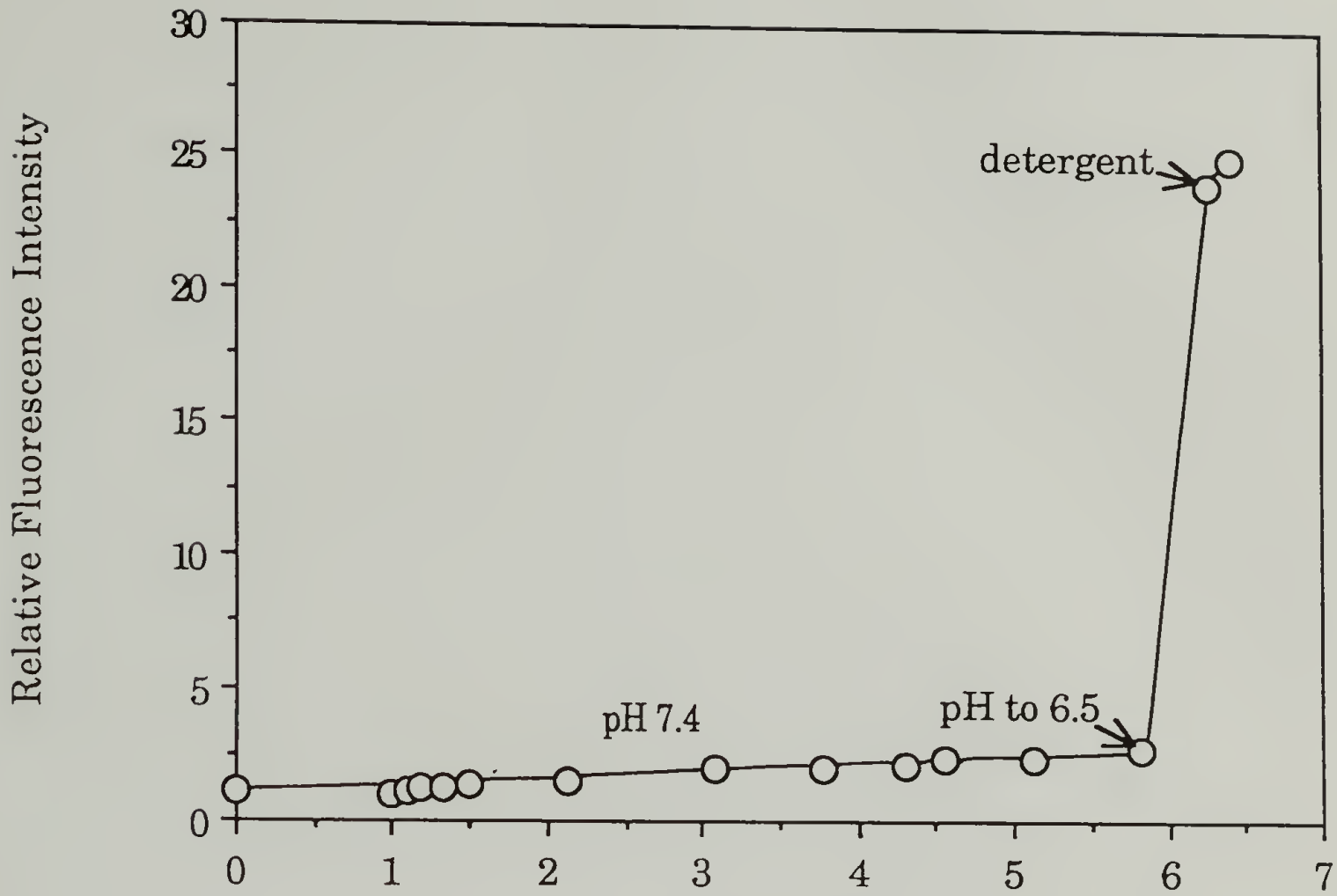


Figure 1.10 Efflux of 6-carboxyfluorescein from sonicated phosphatidylcholine vesicles suspended in 50 mM Tris-HCl, 0.1 M NaCl, 0.03 % PEAA, at indicated pH. Vesicle disruption is detected by monitoring the efflux of entrapped dye through the pH-dependent interaction of PEAA. Acidification of the suspension to pH 6.5 results in near quantitative release of entrapped dye while there is little efflux of dye at pH 7.4. From Tirrell, D. A., Takigawa, D. Y., and Seki, K., Ann. N.Y. Acad. Sci., 446, 237, 1985.

Calorimetric studies revealed that the critical pH for interaction of polymers with DPPC vesicle membranes depended upon the chemical structure and stereochemistry of the poly(carboxylic acid) (66), and the critical pH values for PAA, PMAA, and PEAA were determined to be 4.6, 5.3, and 6.9, respectively, although the precise values would be dependent on the ionic stength of the solution (37). This trend of increasing critical pH values with increasing length of the alkyl side chain corresponds to the trend of increasing apparent pK_a values for this series of polymers (28, 29, 90, 91).

It has been found that as the ionic strength of solution increases, the polyelectrolyte becomes a stronger acid, i.e., the conformational transition occurs at lower pH due to screening of the polymer charge repulsion by ions in solution. Borden et al. investigated the effect of ionic strength on the conformational transition of PEAA using fluorescence spectrometer (37). It was found that as the ionic strength increases the pH at which the conformational transition occurs is shifted to lower values and for example, the critical pH's where inflection points were determined were 6.2 and 6.5 in 0.02 M and 0.40 M phosphate-buffered solutions, respectively (37).

The relationship between the conformational transition of the polyelectrolyte and the structural reorganization of the surfactant bilayer membranes has been examined by Borden et al (37) through the comparison of the pH-dependent changes in fluorescence intensity and those in optical density measurements. It was found that the formation of collapsed polymer chains coincides with the reorganization from a vesicular to a mixed micellar structure.

D. Overview and Goals

Tirrell and coworkers have shown that a polyelectrolyte and phospholipid vesicle membranes combine to form a system that is sensitive to small changes in solution pH. The mechanism of the membrane response has been shown to consist of a structural reorganization of the lipid membranes from a vesicular form at high pH to a mixed polymer-lipid micelle at low pH, the reorganization being driven by collapse of the polymer chain from an expanded form to compact coil upon acidification. It is known that the polyelectrolyte, PEAA, exhibits a pH-dependent conformational transition. Additionally, it was found that the critical pH for the vesicle membrane reorganization corresponded to the critical pH for the conformational transition of PEAA.

The use of macromolecules to effect molecular switching in synthetic bilayer membranes offers substantial advantages in membrane design. One would anticipate, for example, that the pH-dependent conformational transition of PEAA would be subject to modulation by variation in polymer chain structure, (e.g., in tacticity or molecular weight), and that one might exploit such variations to adjust either the critical pH for membrane reorganization or the cooperativity of the structural transition. Tirrell and coworkers investigated the effect of variation of tacticity (64) and molecular weight (38) of PEAA on the critical pH. Calorimetric studies revealed that the critical pH for interaction with DPPC vesicle membranes depends on the chemical structure and stereochemistry. For heterotactic PEAA the observed critical pH was 6.9, while for highly syndiotactic PEAA it was 7.1. Using fluorescence spectrophotometry and optical density measurements,

shifts in the critical pH depending on the molecular weight of polymer were investigated.

From investigation of the interaction of PAA and PMAA with DPPC vesicle membranes, it was found that PAA and PMAA do exhibit pH-dependent complexation with vesicle membranes, but do not cause the catastropic structural reorganization demonstrated with PEAA. Although the critical pH's for PAA and PMAA were measured as 4.6 and 5.3, respectively, they didn't exhibit the structural reorganization to release dye entrapped in EYPC SUV membranes (92). This result is attributed to their weak hydrophobic interaction compared to that of PEAA. Therefore, it would be of interest to synthesize new poly(carboxylic acid)s of useful conformational properties, which can be applied in inducing structural reorganization of phospholipid vesicle membranes at different pH, which gives a wider range of the critical pH. The copolymerization of EAA and MAA will be a good choice for producing a series of poly(carboxylic acid)s. Depending upon the compositions of copolymers, it is anticipated that the critical pH can be controlled between pH 5.3 and 6.9 which are the reported values of those for PMAA and PEAA, respectively. In order to apply copolymers of EAA and MAA in structural reorganization of vesicle membranes, the presence of long blocks of either EAA or MAA in the copolymer would not be desirable. To get useful conformational properties, the random distribution of monomers is the first thing to be checked. Copolymerization of EAA and MAA will be carried out in bulk and in solution, and the copolymers will be characterized by gas chromatography, ^{13}C NMR, and ^{1}H NMR spectroscopy. The information

on the monomer distibution will be obtained by determining the reactivity ratios of monomers.

The copolymers are to be examined in the interaction with DPPC vesicle membranes. Through the investigation on the structural reorganization of vesicle membranes by copolymers of EAA and MAA, if the critical pH is found to be dependent upon the composition of copolymer and the useful shifts in critical pH are observed, we would then consider composition-dependent shifts in critical pH as being attributed to two factors: the different acidities and the different hydrophobic interactions of polymers with varying copolymer composition. If the former is the main reason to cause shifts in critical pH, the origin can be examined by reproducing the same shifts in the critical pH through potentiometric titration experiment. If the same shifts are not observed from titration results, then the different hydrophobic interactions of polymers will be the dominant factor in determining the critical pH in the interaction with DPPC. Thus, the potentiometric titrations of polymers in aqueous solution will be investigated.

With the ability of phospholipid vesicle membranes to release entrapped materials in response to temperature (57, 58) or pH (59), there have been investigations of applications in systems where the pH or temperature changes. It would be of interest then to be able to sensitize vesicle systems to new stimuli as well. There have been some reports of using irradiation as a tool of sensitization (68-72). For most cases the polymer itself was modified to be responsive to irradiation. The diaryliodonium salts, originally developed by Crivello et al.(93-95) as initiators in cationic polymerizations, produce strong acids upon

irradiation in the proper range of wavelength. Using the diaryliodonium salts in changing the solution pH will provide another way of sensitizing PEAA/vesicle system to pH without any modification of polymer. Measurement of the rate of pH depression with varying the parameters such as irradiation time period, the concentrations of polymer, or of iodonium salt in aqueous solution will provide information on the factors controlling the pH depression. After synthesis of the proper diaryliodonium salts, they will be used as proton sources to reproduce the same results in the structural reorganization of the vesicle membranes with release of dye.

In addition to the investigation on the pH-dependent interaction of polymers with phospholipid vesicle membranes, there will be a study about the enhancement of cytotoxicity of immunotoxins by employing PEAA or copolymers. Using the property of copolymers of EAA and MAA in rupturing phospholipid membranes under acidic conditions, but not at neutral pH, this behavior of the copolymers will be used for specific enhancement of cytotoxicity of immunotoxins in a collaboration with Immunogen Inc. (Cambridge, MA). Based on the fact that endocytosis is an obligatory step in cell intoxication by gelonin conjugates (conjugates of single-chain ribosome-inactivating proteins), and endosomes are quickly acidified upon their formation during the conjugation process, one might hope that the copolymers would only destroy endosomal membranes, but not the plasma membranes, and would not be toxic for the cells. If endosomes contain a gelonin conjugate, then, upon the destruction of theendosome, the conjugate will be released into the cytoplasm, where it will be able to reach its target, the ribosome (96, 97).

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CHAPTER II

COPOLYMERIZATION OF 2-ETHYLACRYLIC ACID AND METHACRYLIC ACID

A. Abstract

Copolymers of 2-ethylacrylic acid (EAA) and methacrylic acid (MAA) were prepared in bulk and in N,N-dimethylformamide (DMF). Although precipitation of the copolymers was observed in bulk, the reaction mixtures remained apparently homogeneous in DMF. Best-fit terminal-model reactivity ratios were determined by a non-linear least squares technique to be $r_{MAA} = 1.14$ and $r_{EAA} = 0.23$ in bulk, and $r_{MAA} = 1.91$ and $r_{EAA} = 0.09$ in 50 % DMF solution, respectively. Examination of ^{13}C NMR spectra provided convincing evidence for the formation of statistical copolymers. Copolymerizations richer in MAA provided copolymers of higher molecular weights.

B. Introduction

Copolymerization permits the synthesis of an almost unlimited range of polymers and is often used, therefore, to obtain a better balance of desired properties. In contrast to early investigations on radical copolymerization much experimental data has accumulated showing an

unequivocal effect of the reaction medium in at least some monomer systems. The majority of investigations on the effect of the reaction medium on radical copolymerization deals with reactivity ratios of monomers.

The reactivities of acid monomers are strongly dependent upon the solvents employed since the monomers may exist as dimers or higher aggregates in nonpolar solvents and as species hydrogen-bonded to solvent when polar solvents are employed (1,2). In addition to changes in the intrinsic reactivies of monomers via interaction with solvents, physical phenomena such as reaction mixture heterogeneity are also of importance. The preferential sorption of monomers to precipitated polymers has been reported (3-5) to favor incorporation of polar monomers into copolymers prepared in nonpolar solvents. From copolymerizations of monomers containing polar or ionizable groups capable of hydrogen-bonding interactions, which are influenced strongly by the nature of the reaction medium, Plochocka has drawn attention to the following effects: i). electrostatic repulsion of charged monomers and radicals, ii). changes in monomer polarity (e.g., upon ionization), iii). participation of monomer complexes, iv). hydrogen-bonding of monomers with one another, with the polymer chain, or with solvent, and v). solvent dielectric effects. The copolymerizations of acrylic acid (AA) or methacrylic acid (MAA) with various vinyl monomers have been widely studied (6-11), and the reactivity ratios found in such systems for AA or MAA are higher in nonpolar solvents (e.g., N, N-dimethylformamide (DMF)) (9-14). Harwood and Plochocka have suggested that differential partitioning of polar and nonpolar monomers between free solvent and the domains of propagation

radicals constitutes the principal reason for the large differences in reactivity ratios determined from such copolymerizations in different solvents (10, 15-17).

Although many copolymerizations of carboxylic acid monomers have been reported, we are aware of only two papers (18, 19) that discuss copolymerization between two different carboxylic acids. Chapiro and coworkers examined copolymerization of AA and MAA in bulk and in organic solvents, and reported important medium dffects. We describe in this chapter the analogous copolymerization of EAA and MAA.

C. Experimental

1. Materials

All reagents and their sources are listed below. The reagents were used as received unless indicated otherwise.

Acetone, A.C.S. grade (F)

2,2'-Azobis(iso-butyronitrile) (AIBN) (A) : recrystallized twice from methanol (lit. m.p. 100°C, dec.)

Cycloheptanone (A) : distilled twice (b.p. 42 °C / 3.5 torr)

Deutrium oxide (D_2O) (A)

Diethyl ether (F)

Diethylamine (A)

Diethyl ethylmalonate, 99 % (A)

N,N'-Dimethylformamide (DMF), A.C.S. reagent (A) : stirred with molecular sieves (F) type 3A for 24 hr, dried over powered BaO (A) for 12 hr, and distilled (b.p. 25 °C / 3.8 torr)

N,N'-Dimethylformamide - d_7 (DMF - d_7) (A)

Dimethylsulfoxide - d_6 (DMSO - d_6)

Formaldehyde solution (37 % w/w in water) (A)

Methacrylic acid (MAA) (A) : distilled (b.p. 46 °C / 4.0 torr)

Methanol, A.C.S. grade (F)

Methanol - d_4 (A)

Poly(ethylene oxide) (T)

Sodium Chloride (A)

Sidium hydroxide (A)

Sodium phosphate, dibasic, anhydrous, A.C.S. reagent (A)

Sodium phosphate, monobasic, Gold (A)

Sources

(A) Aldrich Chemical Co. (Milwaukee, WI)

(F) Fisher Scientific (Boston, MA)

(T) Toyo Soda Mfg. Co.

2. Synthesis

a. Synthesis of 2-ethylacrylic acid. 2-Ethylacrylic acid was synthesized by the procedure reported by Ferritto and Tirrell (20). Ethyl ethylacrylate

was purified by distillation prior to hydrolysis to 2-ethylacrylic acid. 2-Ethylacrylic acid was fractionally distilled (b.p.46 °C / 2.8 torr).

b. Radical copolymerization of EAA and MAA in bulk. In a typical experiment, a glass ampule was charged with 0.0034 g (0.5 mol % of monomers) of AIBN, 0.3305 g (3.305 mmol) of EAA, 0.3110 g (3.616 mmol) of MAA, and 0.0620 g (0.5 mmol) of cycloheptanone as an internal standard. A small portion of this mixture was removed and diluted with ca. 9 volumes of methanol for subsequent chromatographic analysis. The ampule was then attached to a vacuum line, subjected to four to five freeze-degas-thaw cycles, and finally sealed under vacuum. The copolymerization was carried out by heating the ampule in a bath maintained at 60 ± 1 °C ; agitation was provided by a magnetic stirring bar. The polymer began to precipitate immediately on warming.

Copolymerizations were run for various time intervals (to conversions of less than 5 %) for the different monomer feeds, and the ampules were removed from the bath and quickly quenched in liquid nitrogen. Reaction mixtures were poured into a large volume of ether with vigorous stirring to precipitate the polymer. The ether solution was stirred for 5 min and the precipitated polymer was separated by filtration and rinsed again with ether. The ether solutions were then combined and analyzed by gas chromatography. Ethereal monomer solutions were analyzed on a Varian 1400 gas chromatograph (6 ft, 1/4 in glass column ; 10 % SP-1000 on 80/100 mesh Supelcoport; flame ionization detection; column 170 °C ; injector 200 °C ; detector 190 °C). The errors in determination of reative amounts of residual monomers were found to be 2-3 %.

c. Radical copolymerization of EAA and MAA in DMF. In a typical experiment, a glass ampule was charged with 0.0079 g (0.5 mol % of monomers) of AIBN, 0.3832 g (3.83 mmol) of EAA, and 0.5036 g (6.23 mmol) of MAA. Sufficient DMF was then added to the mixture to afford a solution containing either 50 % or 75 % DMF. After the freeze-degas-thaw procedure, copolymerizations were run at 60 ± 1 °C for various time intervals (to conversions of less than 5 %) for the various monomer feeds, and the ampules were removed from the bath and quickly quenched in liquid nitrogen. The reaction mixture was dissolved in methanol and the copolymer was precipitated by dropwise addition of the solution into a large excess of ether with vigorous stirring. After separation of the polymer on a fritted glass filter, the precipitation was repeated, and finally the copolymer was rinsed with ether. The copolymer was dried at room temperature, ground to a fine powder, and dried in a drying pistol at 56 °C(refluxing acetone) under reduced pressure. After 48 hours the copolymer was weighed to determine conversion.

3. Measurements

a. NMR spectroscopy. ^{13}C NMR spectra were recorded on a Varian XL-200 NMR spectrometer using a standard single pulse sequence and broad-band H decoupling at various temperatures in the following solvents: Dimethylsulfoxide-d_6 (DMSO-d_6), 100 °C ; D_2O-NaOH, 50 °C ; methanol-d_4, 50 °C . ^1H NMR spectra of copolymers were recorded on a Varian XL-300 NMR spectrometer in DMF-d_7 at 75 °C (see APPENDIX). Spin-lattice relaxation times (T_1) for the methyl carbons of the EAA and MAA units in

the copolymers were obtained at 50 °C in methanol-d_4 on a Varian XL-300 NMR spectrometer by using an inversion-recovery technique (see APPENDIX). The measured T_1 for the methyl carbon of the EAA units was 0.75 ± 0.33 sec : that of the methyl carbon of the MMA units 0.09 sec with negligible error. Quantitative ^{13}C NMR spectra (see APPENDIX) of the copolymers were recorded on a Varian XL-300 NMR spectrometer with gated decoupling to suppress nuclear Overhauser effects and a pulse delay of 12 sec (longer than 5 times the longest methyl group T_1).

b. Molecular weight and viscosity. Molecular weights of the copolymers were determined relative to poly- (ethylene oxide) (PEO) by gel permeation chromatography (GPC) with a set of three columns (TSK 3000 PW, TSK 5000 PW, TSK 6000 PW) and a differential refractometer. Calibration was done using five PEO samples of narrow molecular weight distribution (Toyo Soda Mfg. Co.) with average molecular weights in the range from 18,000 to 996,000 (see APPENDIX). The polymer concentration was about 0.1 wt-% in a phosphate buffer solution (0.036 M, pH = 9.5) that contained 0.33 M NaCl. Inherent viscosities of copolymer samples were measured in a Ubbelohde capillary viscometer (Cannon, 57 / E468) at a concentration of 0.2 wt-% polymer in DMF at 35 °C.

D. Results and Discussion

1. Copolymerization in bulk

Tables 2.1 and 2.2 summarize the results of the copolymerization of EAA and MAA in bulk. These reactions were analyzed by gas chromatography and by ^{13}C NMR spectroscopy, and the two methods are in general in excellent agreement with regard to determination of copolymer composition as a function of monomer feed composition.

Figure 2.1 shows a plot of the mole fraction of MAA in the copolymer versus the MAA mole fraction in the feed, and reveals no systematic differences between compositions determined by the two methods.
The basic hypothesis of simple copolymerization kinetics introduced by Mayo and Lewis (21) and by other groups (22-26) is that the reactivity of an active center only depends upon the monomer unit in the copolymer chain on which the active center is located. Therefore, for a binary copolymerization, the growth of copolymer chains, microstructure development and monomer consumption are uniquely described by four propagating reactions, as follows.

$$\sim\sim M_1\cdot + M_1 \xrightarrow{k_{11}} \sim\sim M_1M_1\cdot$$

$$\sim\sim M_1\cdot + M_2 \xrightarrow{k_{12}} \sim\sim M_1M_2\cdot$$

$$\sim\sim M_2\cdot + M_1 \xrightarrow{k_{21}} \sim\sim M_2M_1\cdot$$

$$\sim\sim M_2\cdot + M_2 \xrightarrow{k_{22}} \sim\sim M_2M_2\cdot \qquad \text{(Equation 2.1)}$$

Table 2.1 Copolymer compositions determined by gas chromatography for the bulk copolymerization of EAA and MAA[a].

Monomer Feed		Mole Fraction MAA		Conversion
MAA(g)	EAA(g)	Feed	Copolymer	(%)
0.0	1.0	0.0	0.0	–
0.0630	0.6159	0.108	0.266	3.3
0.1188	0.5166	0.211	0.417	4.0
0.1681	0.4648	0.296	0.505	5.5
0.2190	0.3909	0.394	0.602	2.5
0.3110	0.3305	0.522	0.670	4.3
0.3414	0.2554	0.609	0.683	3.9
0.3611	0.1922	0.686	0.768	5.3
0.4322	0.1437	0.778	0.816	1.5
0.5001	0.0678	0.896	0.913	2.3
1.0	0.0	1.0	1.0	–

[a] 60 °C, AIBN

Table 2.2 Copolymer compositions determined by ^{13}C NMR spectrometry for the bulk copolymerization of EAA and MAA[a].

Monomer Feed		Mole Fraction MAA		Conversion
MAA(g)	EAA(g)	Feed	Copolymer	(%)
1.8421	19.2776	0.100	0.246	4.2
3.7025	17.1568	0.201	0.351	3.1
11.0562	29.9838	0.300	0.507	2.7
7.3685	12.8520	0.400	0.603	5.2
0.8515	1.0100	0.500	0.664	3.9
12.9027	6.4772	0.697	0.809	4.7

[a] 60 °C, AIBN

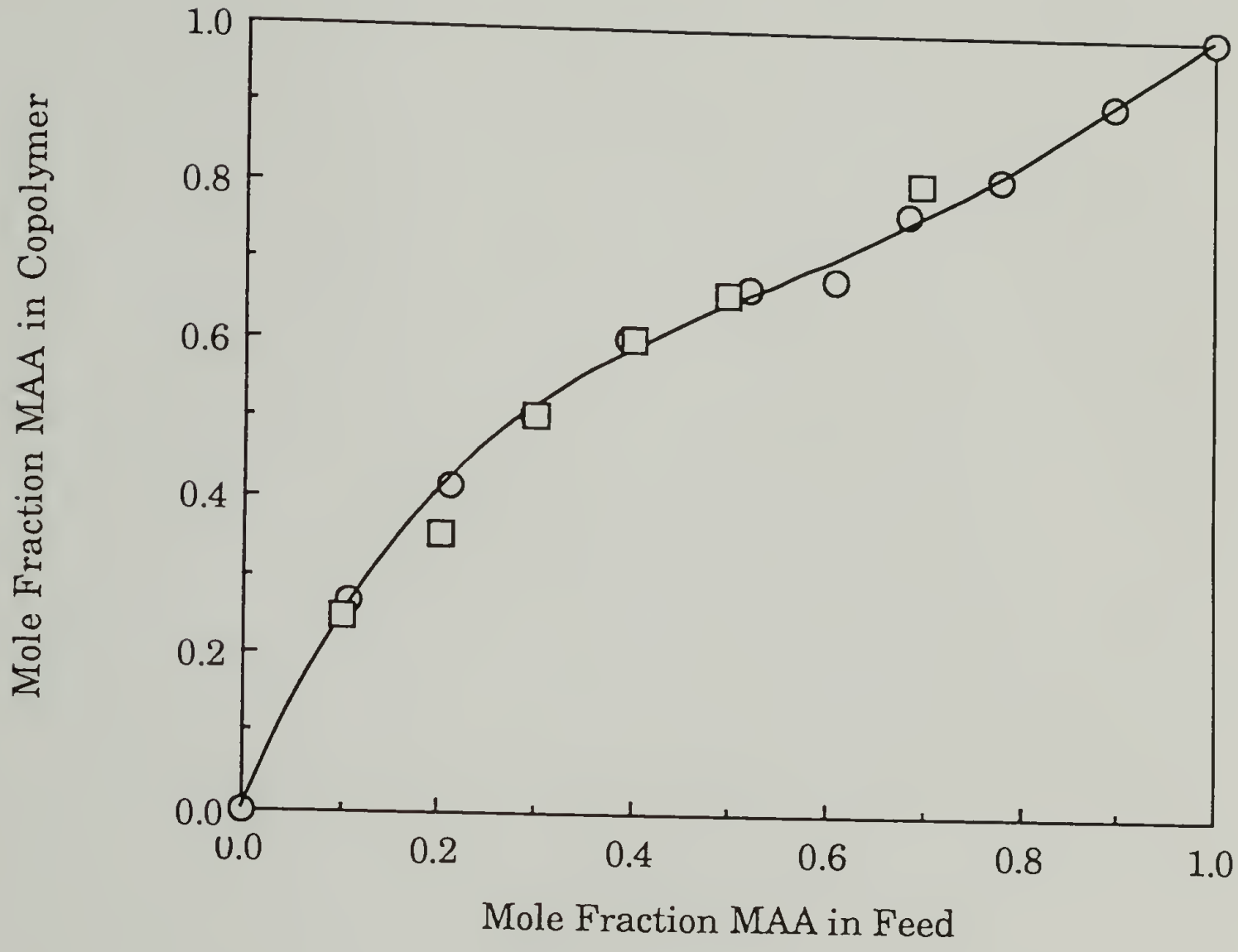

Figure 2.1 Mole fraction MAA in copolymer vs mole fraction MAA in feed. Curve calculated from the terminal-model composition equation with reactivity ratios determined as $r_{MAA} = 1.14$ and $r_{EAA} = 0.23$ for copolymerization in bulk. (○), (□), experimental compositions from gas chromatography and ^{13}C spectrometry, respectively.

The data in Table 2.1 were fit to the composition equation derived for the terminal copolymerization scheme, which relates the instantaneous copolymer composition to monomer feed as follows:

$$m_1/m_2 = (M_1/M_2)\,(r_1M_1+M_2) / (M_1+r_2M_2) \qquad \text{(Equation 2.2)}$$

where r_1 and r_2 are the reactivity ratios of the monomers defined as:

$$r_1 = k_{11}/k_{12} \text{ and } r_2 = k_{22}/k_{21} \qquad \text{(Equation 2.3)}$$

m_1/m_2 the mole ratio of monomers in the copolymer, and M_1/M_2 the mole ratio of monomers in the feed. The best-fit reactivity ratios were estimated by a non-linear least squares technique. The solid line in Figure 1 is a plot of the terminal model composition equation with $r_{MAA} = 1.14$ and $r_{EAA} = 0.23$. The line reproduces the experimentally observed compositions in satisfactory fashion.

Although we have not interpreted the ^{13}C NMR spectra of these copolymers of EAA and MAA in terms of detailed sequence distributions, preliminary examination of such spectra provides convincing evidence for the formation of statistical copolymers. Figure 2.2 shows the carbonyl regions of the spectra of the homopolymers of EAA and MAA, and of a series of copolymers of varying composition. The complexity of these signals, and their variation with composition, argue against the presence of long blocks of either EAA or MAA in any of these copolymer samples. A final note on the bulk copolymerization concerns the heterogeneity of these copolymerization reaction mixtures. Because the EAA-MAA copolymers

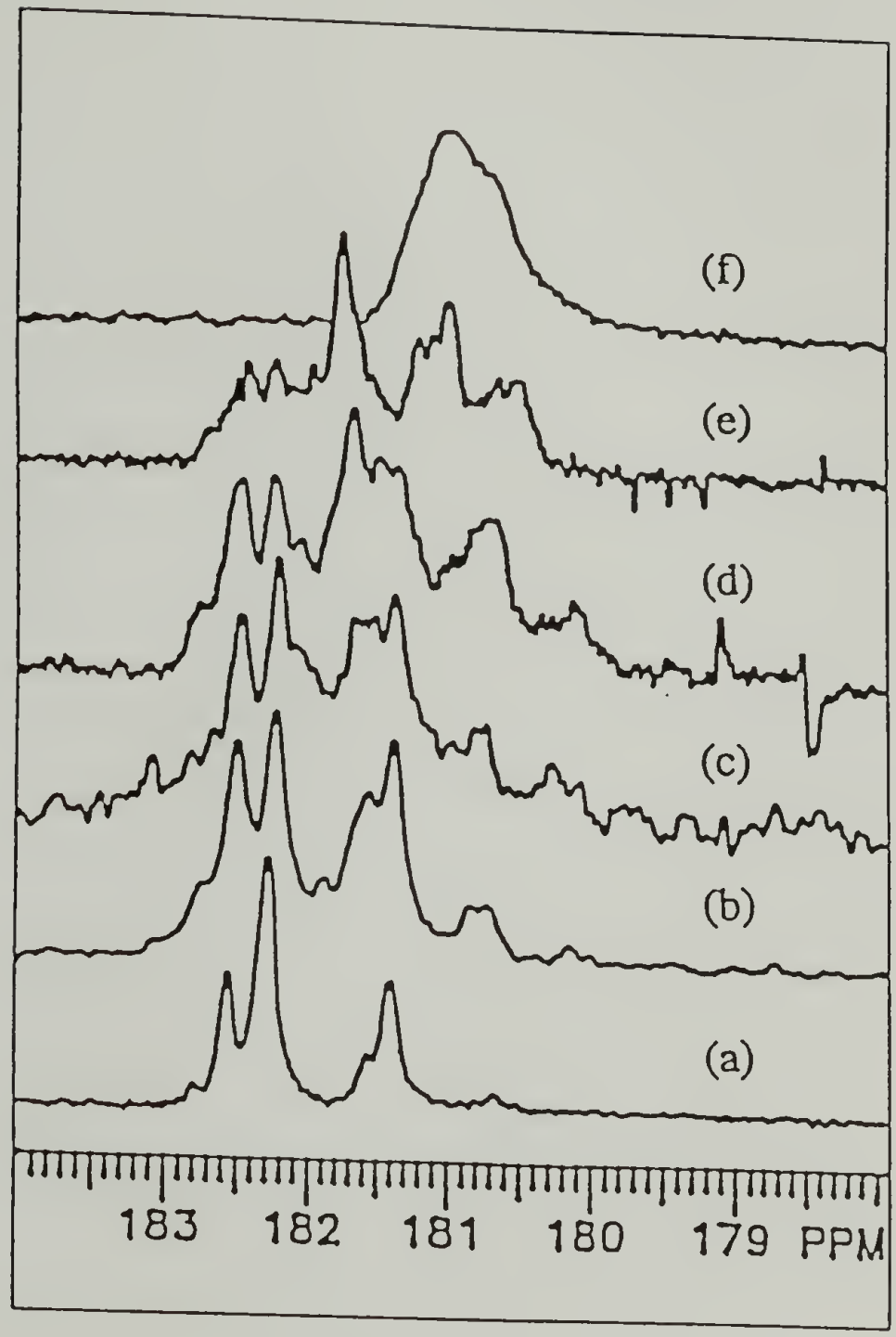

Figure 2.2 ^{13}C NMR spectra of carbonyl regions of a series of copolymers in methanol-d_4 at 50 °C. EAA mole % in feed: (a) 0 (PMAA), (b) 20, (c) 30, (d) 49, (e) 30, (f) 100 (PEAA)

are insoluble in the monomer mixtures from which they are prepared, precipitation was observed in every case, even at low conversion. The copolymerization thus occurs in a two-phase system, and one might then expect differential partitioning of the monomers between the two phases. Harwood (19) has coined the term "bootstrap" copolymerization to describe this phenomenon, in order to emphasize the role played by the growing copolymer chain in determining its own composition. And while precipitation is not a prerequisite for such an effect, differential monomer partitioning would be expected to be most significant in mixtures subject to phase separation. Thus we regard our estimated values of r_1 and r_2 as apparent reactivity ratios which are complex functions of the intrinsic reactivity ratios and the partition coefficients that govern the concentrations of EAA and MAA at the growing chain end.

2. Copolymerization in solution

Table 2.3 summarizes the results of the EAA-MAA copolymerization in DMF. No precipitation was observed in these reactions, and the compositions of the resulting copolymers were analyzed by ^1H NMR spectrometry. Best-fit reactivity ratios for the copolymerization in 50 % DMF solution were estimated by a non-linear least squares technique. Figure 2.3 shows a plot of the mole fraction of MAA in the copolymer versus the MAA mole fraction in the monomer feed, as well as a plot of the terminal model composition equation with $r_{MAA} = 1.91$ and $r_{EAA} = 0.09$. The line again reproduces the experimentally observed compositions in satisfactory fashion.

Table 2.3 Copolymer compositions determined by ^{1}H NMR spectrometry for copolymerization of EAA and MAA in DMF[a].

Monomer Feed		Mole Fraction MAA		Conversion
MAA(g)	EAA(g)	Feed	Copolymer	(%)
0.0	1.0	0.0	0.0	–
0.1272	1.2647	0.105	0.417	3.4
0.1589	0.7339	0.200	0.484	0.7
0.5293	1.4353	0.300	0.610	1.1
0.1705[b]	0.4623	0.300	0.668	3.0
0.3020	0.5416	0.393	0.671	2.0
0.2955[b]	0.5301	0.393	0.734	1.7
0.5900	0.6950	0.497	0.748	4.3
0.5036	0.3832	0.604	0.801	2.1
0.9182	0.4571	0.700	0.849	3.4
1.0346	0.2970	0.802	0.902	3.1
1.0385[b]	0.2925	0.805	0.937	2.7
1.2412	0.1568	0.902	0.947	1.2
1.0	0.0	1.0	1.0	–

[a] 60 °C, AIBN, DMF:monomer 1:1 (w:w)

[b] DMF:monomer 3:1 (w:w)

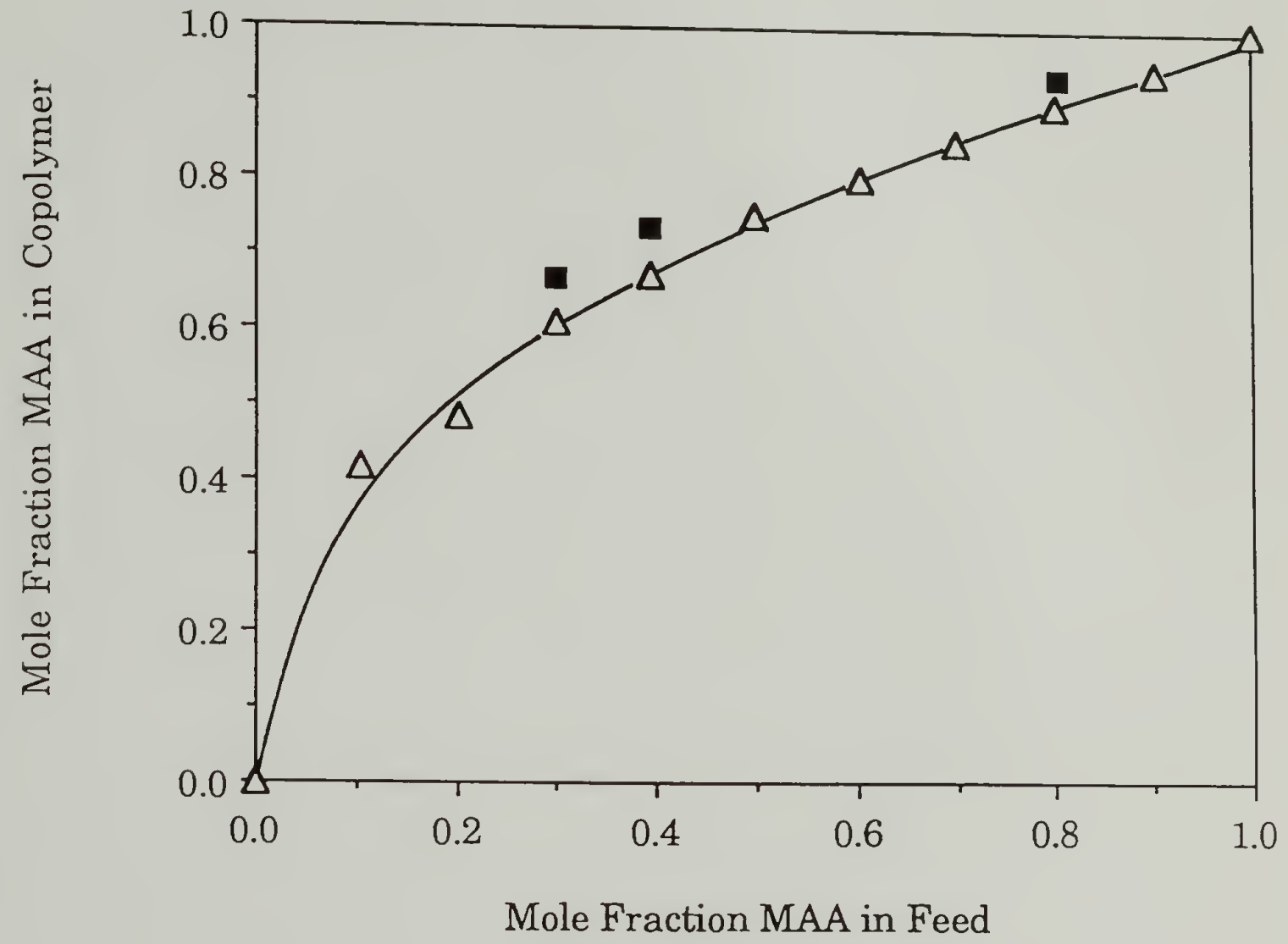

Figure 2.3 Mole fraction MAA in copolymer vs mole fraction MAA in feed. Curve calculated from the terminal-model composition equation with reactivity ratios determined as $r_{MAA} = 1.91$ and $r_{EAA} = 0.09$ for copolymerization in a 1:1 (w:w) mixture of DMF and monomer. (Δ), (■), experimental compositions from ^1H NMR spectrometry (copolymerizations in 1:1 and 3:1 (w:w) mixtures of DMF and monomer, respectively).

The copolymerization results summarized in Figure 2.4 show that the MAA contents of the copolymers produced in DMF were higher than those of copolymers prepared in bulk at the same monomer feed ratios. Furthermore, an increase in the DMF content of the reaction mixture from 50 % to 75 % was accompanied by additional enrichment in MAA. While none of these mixtures was visibly heterogeneous, we regard these results as further evidence for preferential monomer partitioning. We suggest that in bulk and in mixtures relatively poor in DMF, EAA associates strongly with the poorly solvated polymer chain. Addition of DMF solvates the chain more effectively and reduces its selective association with EAA. The reactivity ratios determined for copolymerizations in DMF then reflect more accurately the higher intrinsic reactivity of MAA. Chapiro and coworkers have reported analogous results for the copolymerization of AA and MAA (19). The reactivity ratios of MAA and AA were 2.3 and 0.3, respectively, for copolymerization in bulk, in which the precipitation of polymers was observed (precipitation was also observed in hexane and in 25-50 % methanol solutions where reactivity ratios were found to be similar to those determined in bulk). On the other hand, r_{MAA} and r_{AA} were 6.0 and 0.02, respectively, in 75 % methanol solution, in which the reaction medium was homogeneous. Thus the heterogeneity of the reaction mixture serves to obscure differences in the intrinsic reactivities of the two monomers.Table 2.4 summarizes the molecular weights and inherent viscosities of the EAA-MAA copolymers prepared in the course of this work. In general, copolymerizations richer in MAA resulted in copolymers of higher molecular weight, and bulk copolymerization was superior to solution

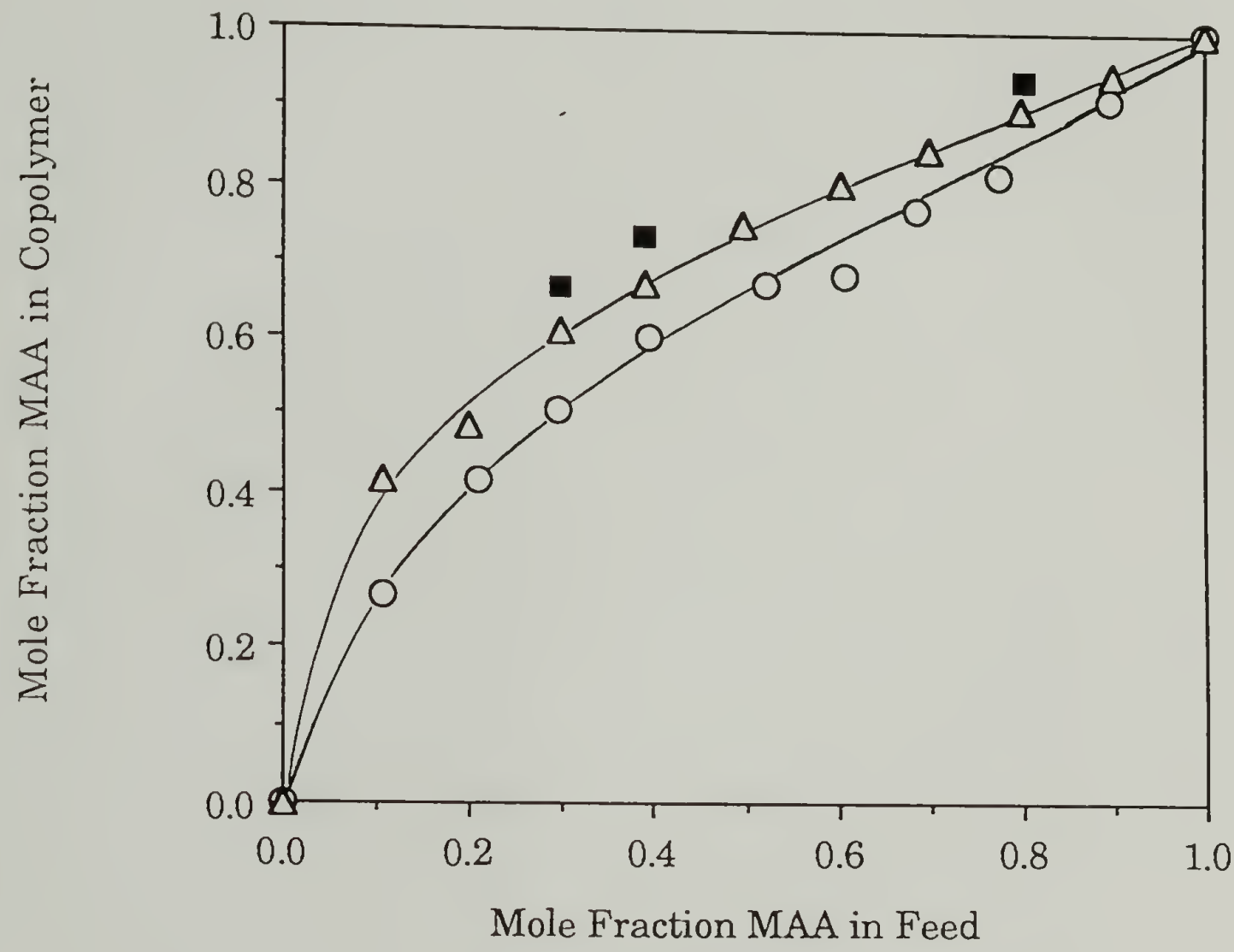

Figure 2.4 Mole fraction MAA in copolymer vs mole fraction MAA in feed(symbols as in Figure 2.1 and 2.3).

Table 2.4 Molecular weights and inherent viscosities of EAA-MAA copolymers.

Mole Fraction EAA in Feed	$\overline{M}_w \times 10^{-4}$[a]		Inherent Viscosities (dL/g)[b]	
	Bulk	DMF[c]	Bulk	DMF[c]
0.9	25.7	–	0.84	0.16
0.8	–	–	1.17	–
0.7	46.7	4.8	1.30	0.27
0.6	–	–	1.65	0.27
0.5	–	–	–	0.33
0.3	58.1	19.7	–	–
0.1	–	25.0	–	–

[a] Relative to poly(ethylene oxide) in aqueous phosphate buffer

[b] 0.2 g/dL in DMF, 35 °C

[c] Copolymerization in 1:1 (w:w) mixture of DMF and monomer

copolymerization in this regard. We have not determined the nature of the reactions responsible for limiting the chain length in these systems.

E. Conclusions

Copolymers of 2-ethylacrylic acid and methacrylic acid were prepared in bulk and in DMF. The reactivity ratios of monomers were estimated by fitting copolymerization data to the composition equation derived for the terminal copolymerization scheme. With increasing amounts of DMF in copolymerization mixtures (from bulk to 75 % DMF solution), copolymers richer in MAA were produced from the same monomer feeds. Partitioning of monomers between the domains of copolymer radicals and the solvent was suggested the origin of this result. ^{13}C NMR spectra provided convincing evidence for the formation of statistical copolymers. Increases in the EAA content of the reaction mixture were accompanied by reduction in the apparent molecular weights of the copolymers.

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CHAPTER III

STRUCTRAL REORGANIZATION OF PHOSPHOLIPID VESICLE MEMBRANES BY COPOLYMERS OF 2-ETHYLACRYLIC ACID AND METHACRYLIC ACID

A. Abstract

A series of copolymers of 2-ethylacrylic acid (EAA) and methacrylic acid (MAA) was used to effect pH-dependent structural reorganization of dipalmitoyl phosphatidylcholine (DPPC) vesicle membranes suspended in aqueous phosphate buffer solutions. Increasing methacrylic acid content in the copolymer shifted the 'critical' pH to lower values, e.g., from 6.55 for PEAA to 5.65 for the copolymer containing 49 mole % EAA. Copolymers of EAA content equal to or less than 40 mole % caused pH-dependent aggregation of DPPC vesicles.

Results of potentiometric titration suggest that the shifts in critical pH should be attributed to composition-dependent changes in the hydrophobic character of the copolymers.

Dye-release experiments showed that complete loss of entrapped material is induced by the most hydrophobic copolymers while partial release of dye was observed for polymers of weak hydrophobicities. A modified PMAA having 14 % of n-hexyl amide side chains induced partial dye release in a relatively wide range of pH. This polymer is not as pH-

sensitive as the other polymers due to the intrinsic hydrophobic nature of n-hexyl side chains regardless of pH.

B. Introduction

An important objective of current vesicle research is the preparation of vesicle membranes of enhanced stability with respect to chemical, osmotic, and mechanical stresses. The need for increased vesicle stability has motivated the preparation of polymerized phospholipid and surfactant vesicles (1-16).

Two elegant stabilization methods have been developed so far; one method is the polymerization of synthetic amphiphiles and the other is blending with polymers. Recently, Kajiyama and coworkers demonstrated composite membranes in which liquid crystal materials (instead of synthetic amphiphiles) are embedded in a polymer matrix. Such composites are applicable to membrane mimetic permeation control, because a distinct change in thermal molecular motion occurs at the crystal-liquid crystal phase transition temperature.

A second objective of vesicle research is the design of membranes with properties that are sensitive to environmental parameters such as temperature or pH (17-19). One might imagine, for example, the use of such vesicles for selective release of drugs in targets of local hyperthermia (17, 18) or low ambient pH (19). Light-sensitive vesicles might release dyes in response to irradiation of a certain frequency or intensity, and

chemically sensitive vesicles might be applied in sensing or diagnostic procedures.

An understanding of polymer-lipid bilayer interactions will allow significant advances in biomaterials development and in the use of phospholipid and surfactant vesicles in control of chemical and biological processes. Tirrell and coworkers have shown that synthetic polymers can be used to render vesicle membranes environmentally sensitive. Environmental sensitivity might be achieved by treatment of the vesicle suspension with a polymer that displays the desired sensitivity.

Interaction of poly(acrylic acid) derivatives with DPPC vesicle membranes has been described previously with the results from differential scanning calorimetry (DSC). The critical pH from the plot of transition width ($\Delta T_{1/2}$) as a function of pH was determined for PEAA of different tacticities, and for PMAA and PAA. It was found that the critical pH depends not only on the chemical structure of the polymer but also on its tacticity (20). Values of the critical pH for interaction with DPPC vesicle membranes were obtained as 4.6, 5.3, and 6.9 for PAA, PMAA, and PEAA, respectively. This result illustrates the effect of chemical structure on the interaction with DPPC. The trend of increasing critical pH corresponds to the apparent pK_a of this series of polymers. From investigation of the tacticity effect on the interaction with DPPC vesicle membranes by preparing PEAA samples that spanned a range of tacticities from 91 % isotactic triads to 88 % syndiotactic triads, the critical pH was 6.9 for heterotactic PEAA vs. 7.1 for highly syndiotactic ones. However, the preparation of those samples caused large differences in polymer molecular weight as well as variations in tacticity. Recently, Tirrell

investigated the molecular weight effect of PEAA on the structural reorganization of DPPC vesicle membranes (21). Three PEAA samples with different molecular weight were prepared and the critical pH's were determined by measuring optical density as a function of pH, and the critical pH was shifted to higher values for the higher molecular weight polymers. The shifts in critical pH were observed and the biggest difference in critical pH values was about 0.3 and the high cooperativity for high molecular weight polymer in structural reorganization was observed compared to low molecular weight one through optical density measurement.

For more useful application, it would be very nice to have new poly(carboxylic acid)s effective in structural reorganization of phospholipid vesicle membranes in wider pH region, rather at more acidic pH. Indeed, it has been shown that sites of primary tumors, metastasis, inflammation, and infection have an ambient pH considerably lower than that of normal tissues (22-28). Copolymerization of EAA and MAA as a candidate for the preparation of new poly(carboxylic acid)s in application with phospholipid vesicle membranes was discussed in CHAPTER II and the formation of statistical copolymers was confirmed, from which useful conformational properties are anticipated. Therefore, the application of copolymers of EAA and MAA in the interaction with phospholipid vesicle membranes is here investigated and the conformational behavior of these copolymers in aqueous solutions will be examined by potentiometric titration.

C. Experimental

1. Materials

All reagents and their sources are listed below. The reagents were used as received unless noted otherwise.

Ammonium thiocyanate (F)

Calcein (A)

Cellulose dialysis tubing (Spectra / Por 6, molecular weight cut off 1,000) (F) : used after being rinsed with methanol.

Chloroform, HPLC grade (F)

1,3-Dicyclohexylcarbodiimide (DCC) (A)

N,N-Dimethylformamide (DMF) A.C.S. reagent (A) : stirred with molecular sieves (F) type 3A for 24 hr, dried over powered BaO (A) for 12 hr, and distilled (b.p. 25 °C / 3.8 torr)

L-α-Dipalmitoylphosphatidylcholine (DPPC), 99 % (S)

Egg yolk phosphatidylcholine (EYPC)

Ferric chloride hexahydrate (F)

n-Hexylamine (A)

Hydrochloric acid, 0.100 ± 0.001 N volumetric standard solution (F)

Sepharose CL 4B-300 (S)

Sodium chloride (A)

Sodium hydroxide, A.C.S grade (A)

Sodium hydroxide, 0.100 ± 0.001 N volumetric standard solution (F)

Sodium phosphate, dibasic, anhydrous, A.C.S. reagent (A)

Sodium phosphate, monobasic, Gold label (A)

Triton X-100, scintillation grade (Am)

Sources

(A) Aldrich Chemical Co. (Milwaukee, WI)

(Am) Amersham Corporation (Arlington Heights, IL)

(F) Fisher Scientific (Boston, MA)

(S) Sigma Chemical Co. (St. Louis, MO)

2. Optical density measurement

DPPC was suspended in 50 mM aqueous sodium phosphate buffer to give a final concentration of 2 mg / 0.8 ml. Deionized water was used throughout. The DPPC suspension was heated to 54 °C for 15 to 20 min and then vortexed for 3 min. Heating and vortexing were then repeated. Copolymer was dissolved in 50 mM aqueous sodium phosphate buffer to give a final concentration of 3 mg / 0.8 ml, and the polymer solution was filtered through a 0.2 μm pore size membrane filter (Millipore). By combining the polymer solution with the DPPC suspension, a sample of 1 : 1.5 of DPPC and polymer was prepared for optical density measurement. The pH was controlled by adding aqueous HCl or NaOH solutions. Each mixture was then heated again to 54 °C for 3 hr and then cooled to room temperature. Optical densities were measured on a Beckman DU-7

UV/VIS spectrophotometer at wavelength (λ) = 500 nm. The temperature of the sample holder was maintained at 28 ± 0.5 °C.

3. Phase-contrast light microscopy

DPPC MLV were prepared by the method described above. A drop of each vesicle preparation was then observed at room temperature by phase-contrast optical microscopy (Olympus BH2, Marcon Instrument Co., Inc.).

4. Potentiometric titrations

a. Sample preparation. PEAA (10 ± 0.1 mg, 0.1 mM repeating units) was dissolved in 1 ml of 0.100 ± 0.001 N standardized NaOH. The polymer solution was then combined with 9 ml of 100 mM NaCl aqueous solution, resulting in 1 mg / ml of the final concentration of polymer in solution. Copolymer samples were prepared similarly.

b. Measurements. The sample was placed in a jacketed beaker connected to a Brinkmann Lauda RM-6 circulating bath and the temperature was maintained at 25 ± 0.2 °C. Argon was slowly bubbled through the sample for 10 minutes with stirring, and an argon blanket was maintained above the sample solution throughout the titration. The titration was carried out with the injection of 5, 10, 20, or 30 µl aliquots of 0.100 ± 0.001 N standardized HCl solution from a 25 µl syringe to the stirred sample. The pH was determined about 10 minutes after stopping the stirring, when almost no fluctuation in pH was observed. The solution pH

was measured to within 0.01 pH units after each injection with a Corning Model 155 ion analyzer equipped with an ultra-thin micro-electrode (Aldrich, Hg /Hg_2Cl_2, pH combination electrode). The pH meter was calibrated before titration with standardized buffers at pH 4, pH 7, and pH 10.

5. Release of calcein from EYPC SUV

a. Synthesis of PMAA derivative with n-hexylamide. PMAA (0.0868 g, 1.0 mmol) was dissolved in anhydrous DMF. DCC (0.0424 g, 0.2 mmol) was added to the solution, followed by the addition of n-hexylamine (31 µl, 0.3 mmol) after stirring for 1 hr. The reaction mixture was then stirred at 24 ^{o}C for 48 hr and added to ethyl acetate to precipitate the polymer. The precipitate was filtered and then dissolved in methanol. Precipitation was again carried out in ethyl acetate, and the polymer was was isolated by filtration and dissolved in methanol. The resulting solution was filtered and the volume of the filtrate was reduced to about 10 ml on a rotary evaporator. The solution was placed in cellulose dialysis tubing (Spectra /Por 6, 1000 MW cutoff) and dialyzed against methanol for 48 hr. The external methanol was exchanged every 12 hr. The polymer was precipitated into ether by dropwise addition, filtered, and then dried under vacuum. The polymer was found to contain 14 mol % of n-hexylamide linkages (based on PMAA repeating units) from elemental analysis. Anal calcd for this composition: C, 57.99 ; H, 8.51 ; N, 2.00 % ; O, 32.50Found: C, 54.59 ; H, 9.86 ; N, 1.84 % ; O, 33.71. Elemental analysis was done at the Microanalysis Laboratory at the University of Massachusetts at Amherst.

b. Preparation of vesicle samples. A 200 mM solution of calcein in 10 mM tri-HCl (100 mM NaCl), pH 10.12 was added to a long-neck round bottomed-flask containing a thin film of EYPC, and brief vortex mixing was carried out to give a turbid MLV suspension (40 mg EYPC / 2.7 ml). The resulting suspension was then sonicated in an ice-water bath with a Branson Model 185 Cell Disruptor equipped with a 1/8 inch diameter titanium microtip at a power of about 35 watts. The sonication was continued for 10 min and then stopped for about a minute to cool down the tip. The same procedure was repeated until a transparent solution was obtained. The sonicated sample was then centrifuged for 30 minutes in an IEC Model CL clinical centrifuge with a 4 x 50 ml swinging bucket rotor at setting 5 (~2500 rpm) to sediment large multilamellar vesicles and titanium particles. Small vesicles in the supernatant were then applied to a gel filtration column (Sepharose CL-4B 300, 2 x 25 cm). Vesicle fractions of elution volume ca.18 to 31 ml were collected and stored at 5 °C. The concentration of the lipid was determined to be about 0.38 mg / ml by the method of Stewart (28), as follows. To the separated vesicle fraction was added 2.00 ml of 0.1 N ammonium ferrothiocyanate solution. The mixture was vortexed, 2.000 ml of chloroform was added, and the mixture was vortexed again. After a few minutes, to allow for clear separation, the chloroform phase was withdrawn and the absorbance at 488 nm was measured on a Beckmann DU-7 UV/VIS spectrometer. Calibration curves (see Appendix) were prepared by repeating this procedure on a series of samples of known lipid concentration.

c. Measurements. Stock solutions of each polymer (2 mg / ml) in 10 mM tris-HCl (100 mM NaCl), pH 10.12 were prepared. The polymer solution was added to the suspension of calcein-loaded vesicles to give a 1 mg / ml polymer concentration. Fluorescence intensity (520 nm) as a function of time was recorded on a Perkin-Elmer MPF-66 fluorescence spectrometer equipped with a thermostatted sample compartment. An excitation wavelength of 495 nm and 5 nm slit widths were used. The maximum fluorescence intensity from each sample was determined by adding 0.05 ml of 15 % Triton X-100. Temperature was maintained at 25 ± 0.2 ^{o}C with a Brinkmann Lauda RM-6 circulating bath. Correction factors for the wavelength-dependent intensity of the xenon lamp of the MPF-66 were generated using Rhodamine B (3 g / l in ethylene glycol) as a quantum counter over an excitation wavelength range from 200 nm to 600 nm. Correction factors for the wavelength-dependent efficency of the MPF-66 detection systems were also generated from the Rhodamine B calibrated spectral output of the xenon lamp. Fluorescence spectra were automatically corrected with the Perkin-Elmer CLS software supplied with the MPF-66.

D. Results and Discussion

1. Turbidity measurements

The reorganization of DPPC from vesicular aggregates into mixed polymer-lipid micelles is accompanied by a large decrease in average aggregate size (30, 31), and simple turbidity measurements provide a

convenient means to investigate this transition. Fig 3.1 shows the pH-dependent turbidities of 1:1.5 (by weight) aqueous mixtures of DPPC with PEAA, with each of the copolymers of EAA and MAA, and with PMAA. Polymer-free DPPC suspensions are
uniformly turbid over the pH range 7.5 to 5.5. In contrast, lipid mixtures containing PEAA or EAA/MAA copolymers clarify upon acidification, with the transition found at pH 6.55 ± 0.03 for PEAA, at 6.25 ± 0.03 for the 75 mol% EAA copolymer, at 5.80 ± 0.03 for the 65 mol% EAA copolymer and at 5.65 ± 0.03 for the 49 mol% EAA copolymer.

Although precise measurements have not been made, it appears that as the EAA content in the copolymer is increased, the vesicle-to-micelle transition becomes somewhat sharper. For example, the slope of the turbidity curve for PEAA is steeper than that for the copolymer of 49 mol % EAA content. Decreasing EAA content in the copolymer shifted the clarification of the vesicle suspension to lower pH and slowed the kinetics of the process. These results are tentatively attributed to composition-dependent changes in the hydrophobic character of these polymers. This point will be discussed in detail below.

Transient aggregation was noted in each of the samples in the range of pH from 6.9 to 7.2 at 54 °C after 20 min of incubation. In this pH range ca. 60 % of the polymer-bound carboxylic acid groups are protonated in PEAA and in each of the copolymers as shown below (see Figure 3.8). These aggregates disappeared on gentle vortex mixing, and the final suspensions were uniformly turbid.

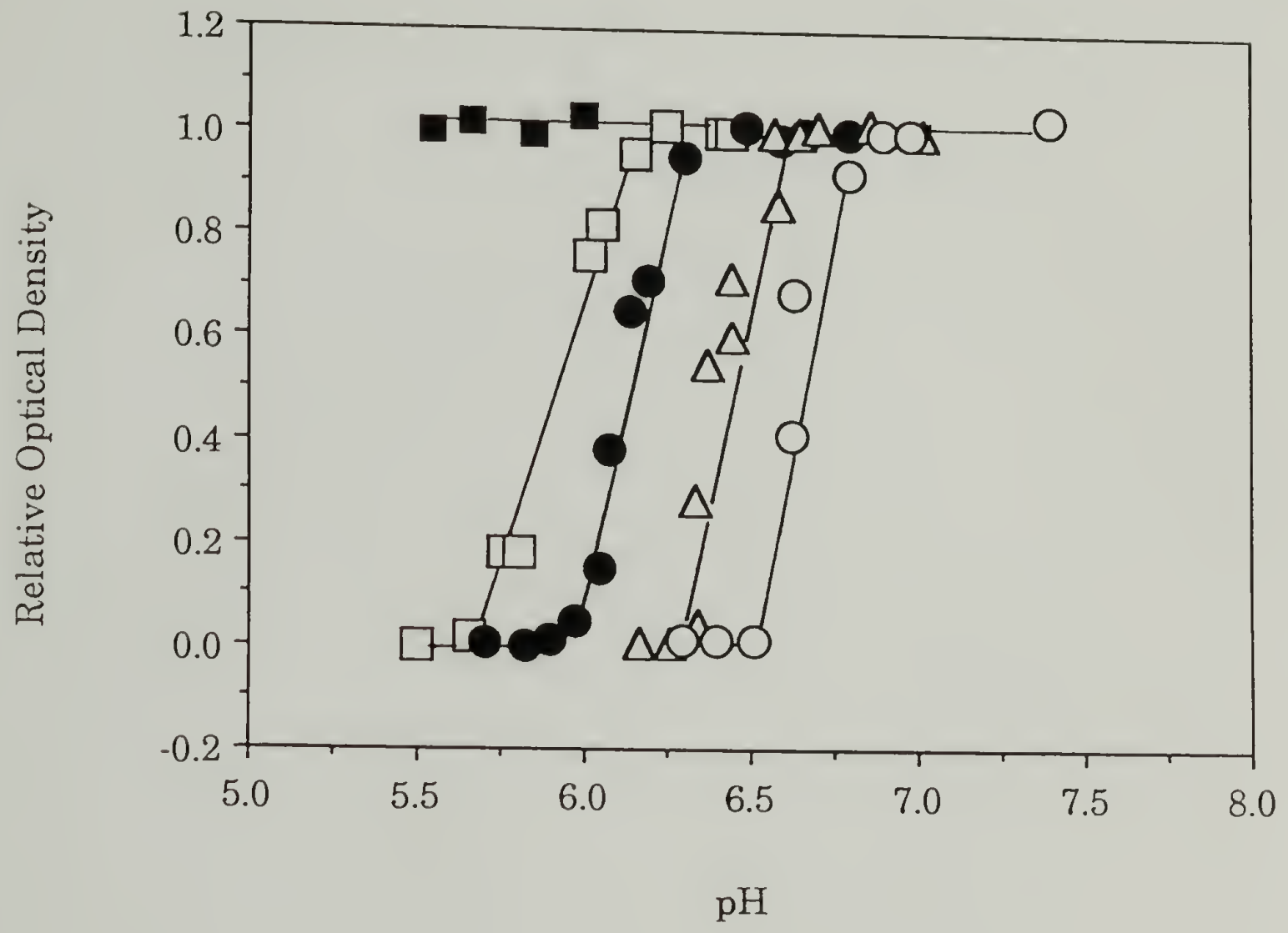


Figure 3.1 Optical density (relative to optical density at pH 7.0) at 500 nm of 1:1.5 DPPC/polymer mixtures in 50 mM aqueous phosphate buffer solutions as a function of pH; (○), PEAA; (△), 73 mol % EAA; (●), 58 mol % EAA; (□), 49 mol % EAA in copolymer; (■), PMAA. Estimated errors are about ± 0.02 in pH and relative optical density.

Samples prepared from copolymers of EAA content equal to or less than 40 mol% showed more complicated behavior upon reduction of pH. For the copolymer of 40 mol % EAA content, acidification to pH about 5 converted the uniformly turbid suspension into a mixture of aggregates (apparently consisting of both lipid and polymer) and clear solution. These aggregates were quite stable and survived even vigorous vortexing. The copolymers showing this behavior were completely soluble under the same conditions in the absence of lipid (they were soluble in aqueous phosphate buffer even at pH 2.0) and polymer-free vesicle suspensions do not aggregate in this range of pH and ionic strength. When the pH of these samples was then increased by adding NaOH , the aggregates disappeared and suspensions of uniform but reduced turbidity were obtained. Clarification was faster and more complete when the pH was raised to higher values (pH between 5.2 and 6.0). At ca. pH 6, the aggregates disappeared and a slightly turbid suspension (much less turbid than the original vesicle preparation) was obtained, whereas upon adjustment of pH to 6.2, completely clear solutions were obtained. In contrast, when the pH of the initially prepared samples was reduced directly from 7.6 to 6.2, no changes in turbidity were observed.

We offer the following tentative explanation for this complex behavior. As the pH is lowered to ca. 5 copolymer-DPPC aggregates begin to form. At around pH 5, the rate of aggregation increases, owing to the increased protonation of the polymer. It has been suggested by Tirrell and coworkers (20) that the initial interaction of PEAA with DPPC vesicle membranes occurs via hydrogen-bonding between the protonated carboxylic group and the phosphate head group of DPPC. Once this initial binding occurs, either

of two possibilities may follow: i) if the polymer chains are hydrophobic enough to solubilize the vesicle membranes into mixed micellar structures, the optical density is reduced, i.e., behavior similar to that of PEAA would is observed., or ii) if the polymer is not sufficiently hydrophobic, bridging of membrane surfaces occurs, leading to aggregation. The resulting aggregates are apparently stable with respect to reorganization into mixed micelles, such that micelles are formed only upon disaggregation in the presence of base (pH≥6.2). The fact that reorganization does not occur upon direct acidification to pH 6.2 suggests that there are important kinetic effects in these systems, and it is not clear whether micellization or vesiculation is rate limiting.

2. Light microscopy

The aggregation behavior of these polymer-lipid mixtures was examined by phase-contrast optical microscopy. Phase-contrast light microscopy is routinely used to monitor lipid dispersions, and differences in the refractive indices of lipid and water even allow discrimination between oligolamellar vesicles (low phase-contrast spheres) and multilamellar vesicles (phase-dark spheres) (34). Figures 3.2 and 3.3 show micrographs of mixtures of PEAA and DPPC vesicles at pH 7.6 and pH 6.3, respectively. In the sample of higher pH (and in polymer-free DPPC samples) well dispersed vesicles are observed. But in the sample of pH 6.3 no vesicle structure is apparent. Borden et al have used transmission electron microscopy to investigate the size and morphology of the membrane reorganization products at low pH. They found in such samples extended stacks of disk-like structures 54 ± 6 Å in thickness and 160 ± 50 Å

Figure 3.2 Phase-contrast micrograph of the mixture of DPPC multilamellar vesicles and PEAA in 50 mM phosphate buffer at pH 7.6. The observation was made at room temperature (bar = 10 μM) after heating the sample at 54 °C for 5 minutes (DPPC and PEAA, 1 mg and 1.5 mg/ml, respectively).

Figure 3.3 Phase-contrast micrograph of the mixture of DPPC multilamellar vesicles and PEAA in 50 mM phosphate buffer at pH 6.3. The observation was made at room temperature (bar = 10 μM) after heating the sample at 54 °C for 5 minutes (DPPC and PEAA, 1 mg and 1.5 mg/ml, respectively).

in diameter (35). These small micellar aggregates would not be revealed by phase-contrast optical microscopy. Figures 3.4 and 3.5 show phase-contrast micrographs of DPPC-PMAA mixtures. At high pH, normal vesicles are observed, but when the pH is reduced to 5.0, large aggregates are observed (Figure 3.5).

3. Copolymer composition effects

As the EAA fraction in the copolymer is reduced, the critical pH for membrane reorganization is shifted to lower pH. A plot of the critical pH as a function of EAA mole fraction in the copolymer is shown in Figure 3.6. The difference in the values of the critical pH for PEAA and for copolymer of 49 mol% EAA is about 0.9 pH units. This shift might be attributed to variation either in the acidity or in the hydrophobic character of the copolymers. In order to investigate the origin of the shifts, the potentiometric titration behavior of the copolymers was examined. If the titration curves and critical pH shift in similar fashion, composition-dependent changes in acidity may be regarded as dominant. Departure from this behavior is most reasonably attributed to changes in hydrophobicity.

For polyelectrolytes, as the extent of ionization increases, it becomes increasingly difficult to remove protons from the charged chain, and the apparent pK_a rises (36-38). For many polyelectrolytes, e.g., poly(acrylic acid), as the degree of ionization increases there is a continuous increase in the dimensions of the polymer chain due to charge repulsions (39). For PMAA and PEAA, however, there is resistance to chain expansion, which originates from hydrophobic interaction between the alkyl side chains (36,

Figure 3.4 Phase-contrast micrograph of the mixture of DPPC multilamellar vesicles and PMAA in 50 mM phosphate buffer at pH 7.6. The observation was made at room temperature (bar = 10 μM) after heating the sample at 54 °C for 15 minutes (DPPC and PMAA, 1 mg and 1.5 mg/ml, respectively).

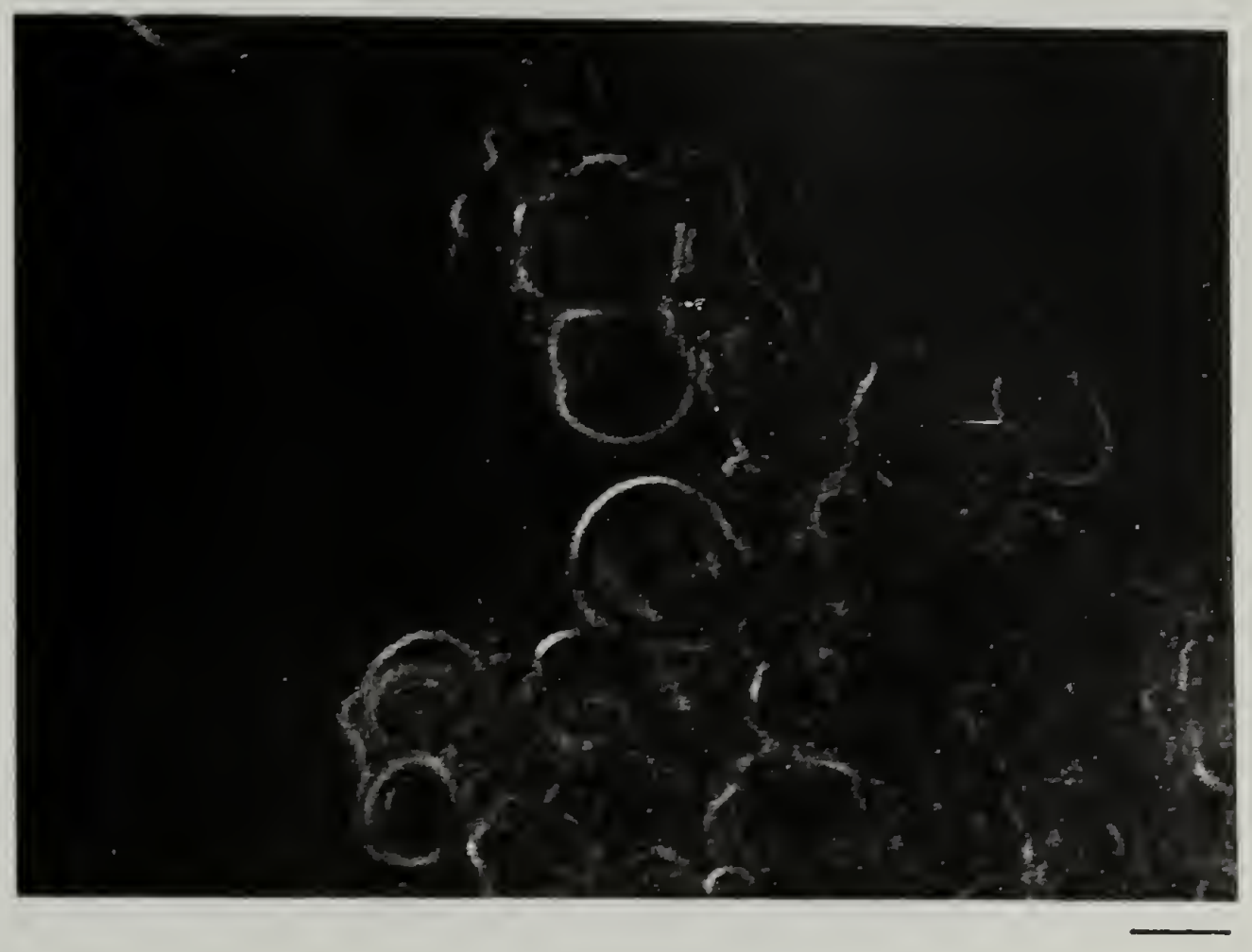


Figure 3.5 Phase-contrast micrograph of the mixture of DPPC multilamellar vesicles and PMAA in 50 mM phosphate buffer at pH 5.0. The observation was made at room temperature (bar = 10 μM) after heating the sample at 54 °C for 15 minutes (DPPC and PMAA, 1 mg and 1.5 mg/ml, respectively).

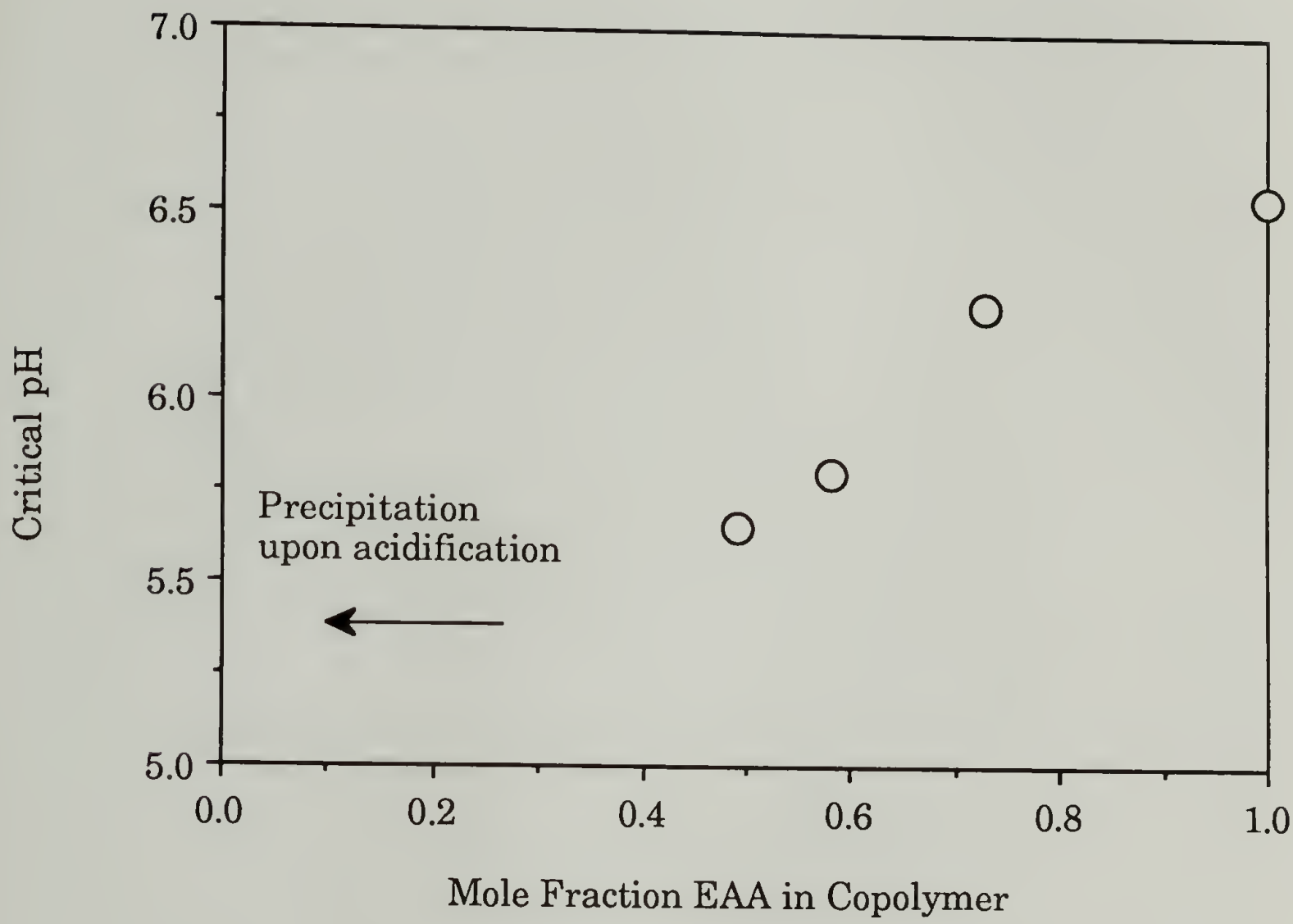


Figure 3.6 Critical pH for clarification of 1:1.5 DPPC/polymer mixtures in 50 mM aqueous phosphate buffer solutions as a function of EAA mole fraction in the copolymers.

40-45). These hydrophobic interactions keep the polymer chain in a compact globular conformation at low degrees of ionization in aqueous solution. The alkyl side chains in the interior of the globular structure are protected from contact with water, while the ionized carboxyl groups on the exterior of the structure are solvated by water molecules. At a certain degree of ionization the resistance to chain expansion is overcome by charge repulsion, and the polymer chain undergoes a conformational transition from a compact to an expanded coil. This conformational transition has been observed as a discontinuity or plateau in a plot of the degree of ionization as a function of pH or pK_a (40-52).

Potentiometric titrations of PEAA, and of the MAA copolymers of 65 and 49 mole % EAA content were carried out in 100 mM NaCl aqueous solutions (1 mg PEAA / mL). The change in pH for a solution of PEAA with added HCl is shown in Figure 3.7.

The degree of ionization, α, is calculated from the electroneutrality condition (53) :

$$\alpha = \frac{C_{Na^+} + C_{H^+} - C_{OH^-} - C_{Cl^-}}{C_M} \qquad \text{(Equation 3.1)}$$

where C_{Na^+}, C_{H^+}, C_{OH^-}, and C_{Cl^-} represent the concentrations of sodium, hydrogen, hydroxyl, and chloride ions, respectively, and C_M is the monomer repeating unit concentration. Plots of α versus pH are shown in Figure 3.8 for PEAA and for the copolymers of 49 and 65 mol% EAA content.

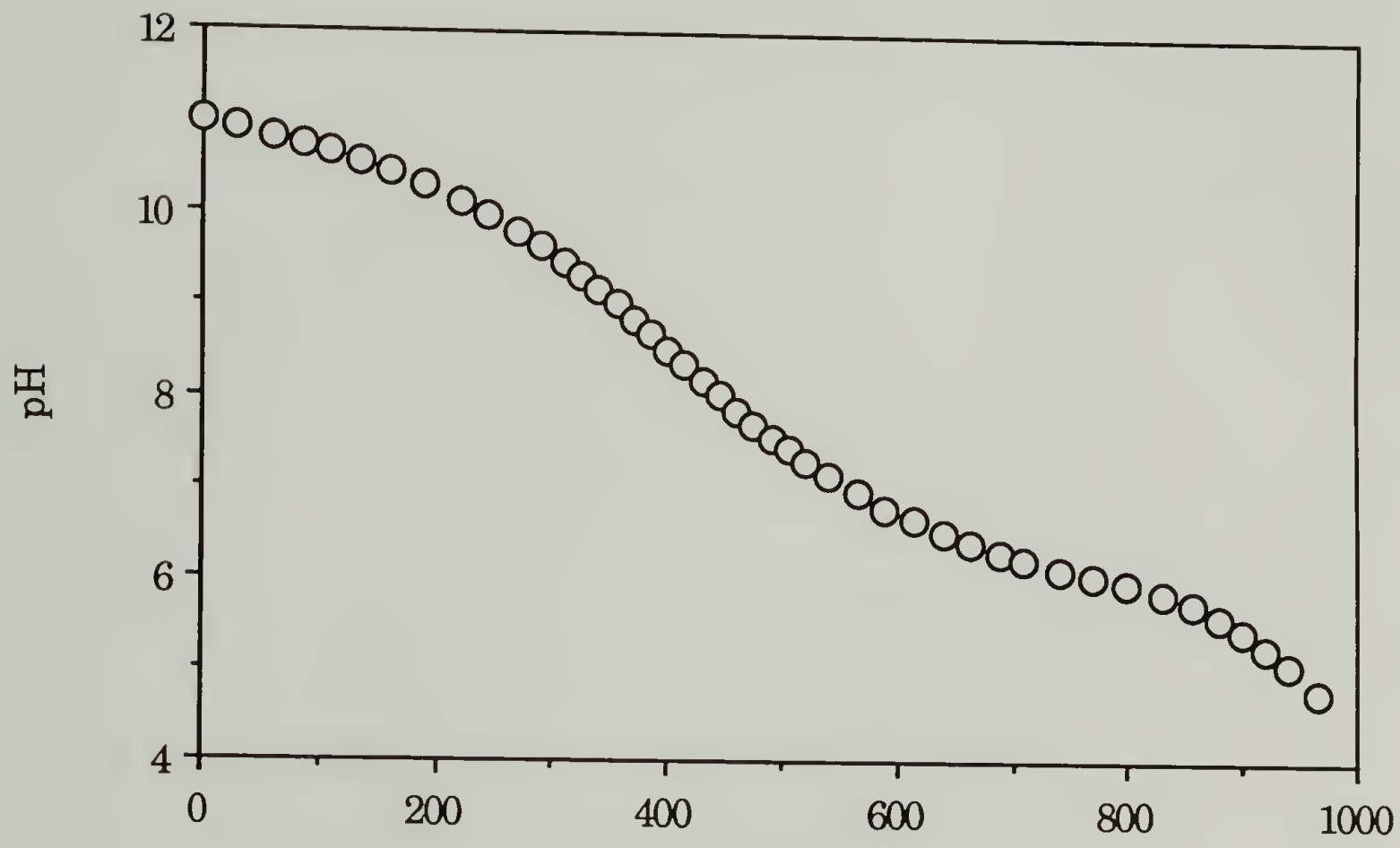


Figure 3.7 pH of polymer solution (PEAA 1 mg/ml and 100 mM NaCl) as a function of added 0.100 N HCl at 25 ± 0.2 °C.

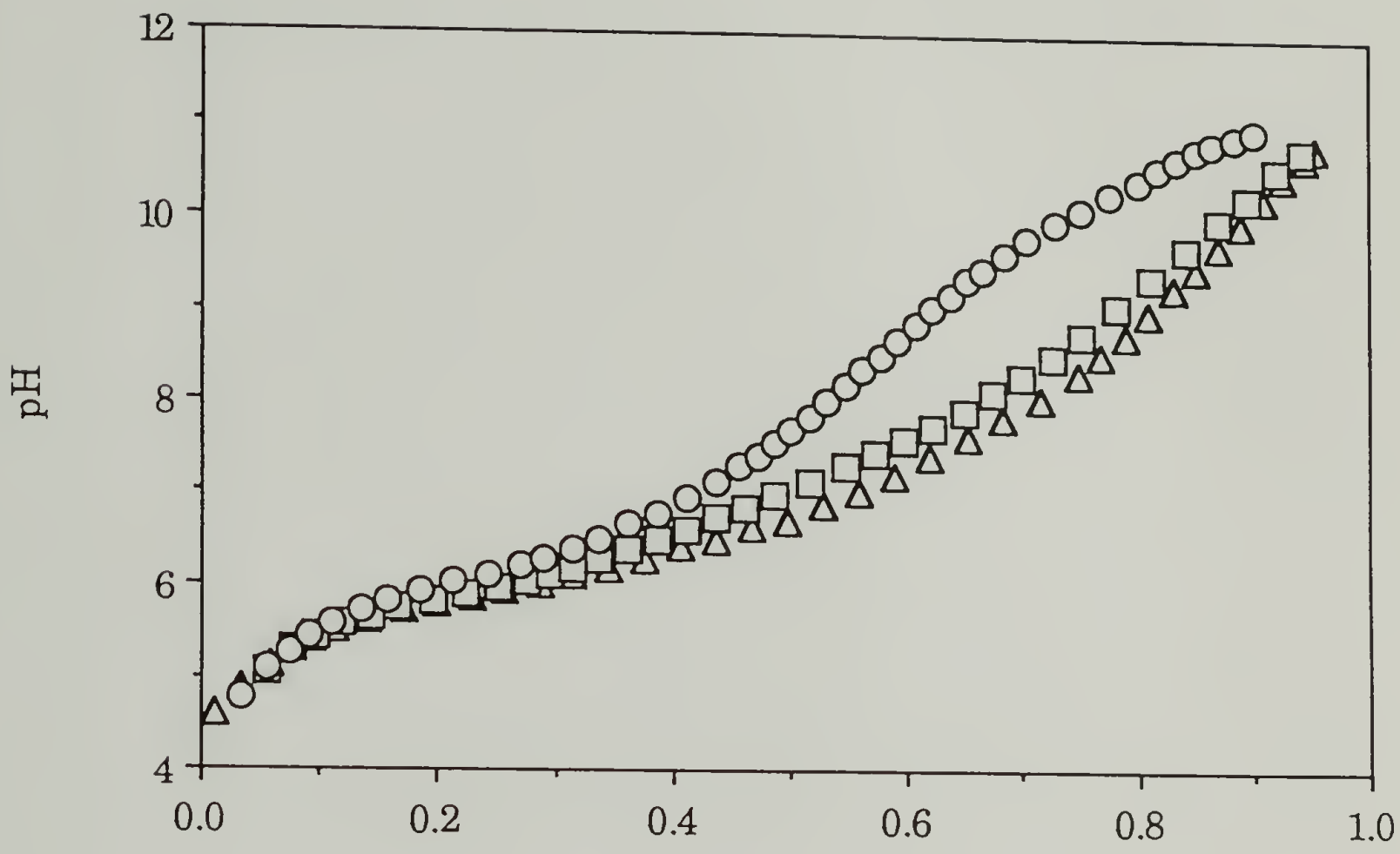


Figure 3.8 pH as a function of the degree of ionization in polymer solutions (1 mg/ml and 100 mM NaCl) at 25 ± 0.2 °C. (○); PEAA, (□); 65 mol % EAA, (△); 49 mol % EAA in copolymer.

It is clear from the similarities in these curves, particularly near pH 6, that changes in acidity cannot be the primary origin of the composition-dependent shifts in critical pH for membrane reorganization by these polymers. Increasing hydrophobic character must play an important role, since membrane-reorganization clearly occurs at larger α for copolymers of increasing EAA content.

If we assume that the potentiometric titration curves of the compact and expanded forms existing in the transition region are the same as those of completely compact and expanded molecules, respectively, it is possible to calculate the fraction of each conformation via the method of Nagasawa and Holtzer (54). The method is graphically outlined in Figure 3.9 for a sample of PEAA. Curve I is the measured titration curve, while curves II and III are the presumed titration curves of completely compact and expanded molecules, respectively, as obtained from extrapolation. The extrapolation for curve III is relatively reliable; however, that from curve II is uncertain owing to the small number of data points used. The system at point 'a' has an average degree of ionization α_a, and is assumed to consist of a mixture of compact chains or chain sements of degree of ionization α_c and expanded chains of degree of ionization α_e. In this manner, for any actual degree of ionization, the degrees of ionization of the compact and expanded fractions can be estimated. The fraction of PEAA in the compact chain conformation (F_c) is then calculated as:

$$F_c = \frac{\alpha_e - \alpha_a}{\alpha_e - \alpha_c} \qquad \text{(Equation 3.2)}$$

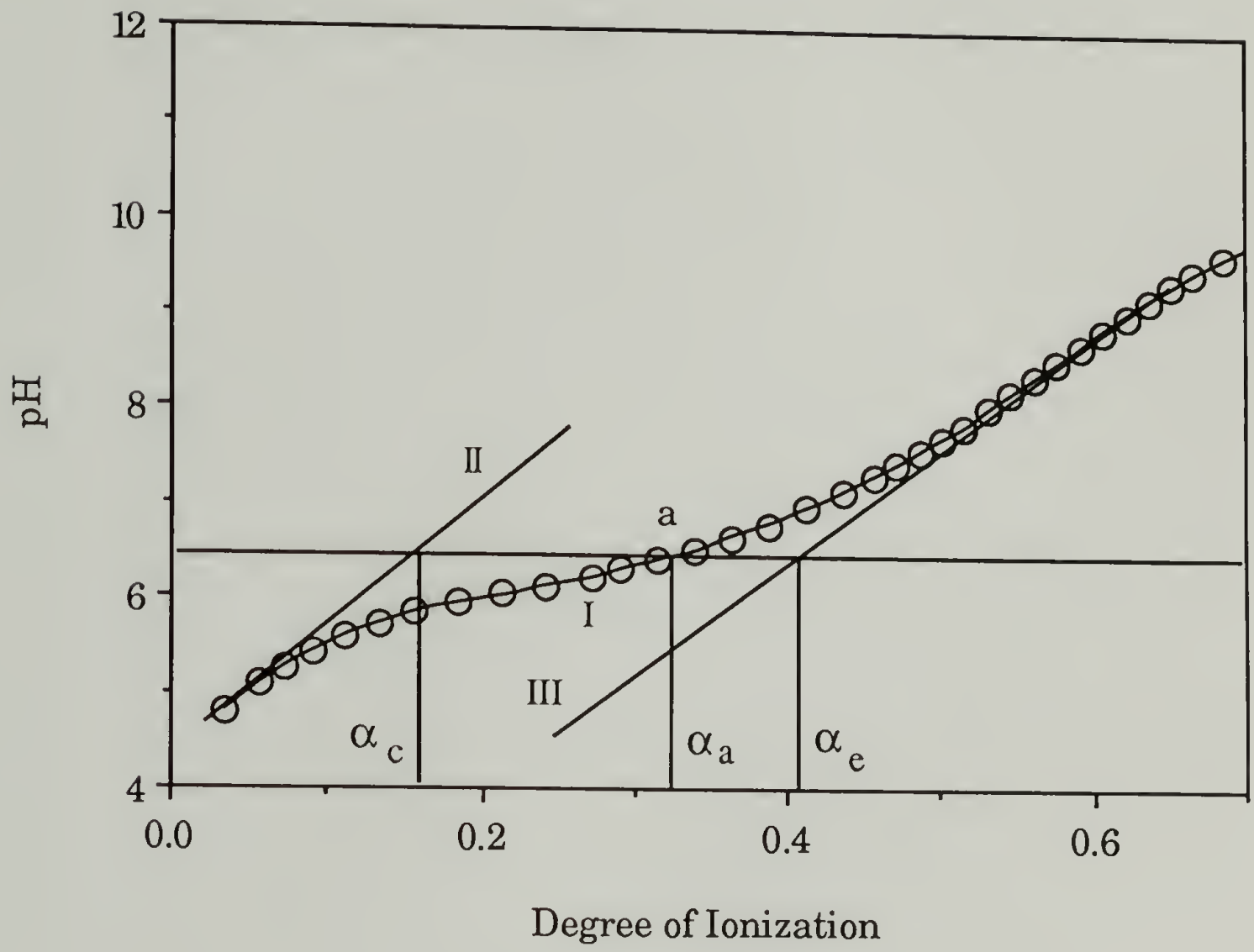


Figure 3.9 Determination of the fraction of collapsed coils (Fc) in the conformational transition region of PEAA. From Nagasawa, M. and Holtzer, A., J. Am. Chem. Soc., 86, 538, 1964.

The plots of compact fraction as functions of pH and the degree of ionization are shown in Figures 3.10 and 3.11, respectively. From Figure 3.10 we see that above pH 7.2 there are substantially no compact coils for PEAA, and that the compact fraction rises most abruptly near pH 6.2. The copolymers show similar behavior, although among these polymers the increase in compact fraction in the transition region is faster for PEAA than for the copolymers. The compact fractions of PEAA and of the 49 mol % copolymer at the critical pH determined previously are found to be about 0.33 and 0.8, respectively. This interpretation again illustrates the different hydrophobicities of the polymers ; a higher compact fraction is required for the copolymer to convert vesicles into mixed micelles because the copolymer is less hydrophobic than PEAA. In the plots of compact fraction versus the degree of ionization no simple relationship is observed; the compact fraction of each polymer is almost linearly dependent upon the degree of ionization and similar values of the compact fraction were found for the three polymer samples over the full range of the experiment.

It was of interest to investigate the dye release behavior for vesicle membranes treated with copolymers that are not hydrophobic enough to effect complete membrane reorganization. PMAA itself was examined and substantially no release was observed. PMAA was then modified by providing 14 mol % of n-hexyl amide side chains in place of carboxylic groups (see Figure 3.12). The n-hexylamine itself is not capable of inducing release of dye. Figure 3.13 summarizes the results of dye-release from SUV of EYPC for a series of polymers. The differences in maximum fluorescence intensities noted after adding Triton X-100 are due to quenching of calcein fluorescence below pH 6. Although the results are not

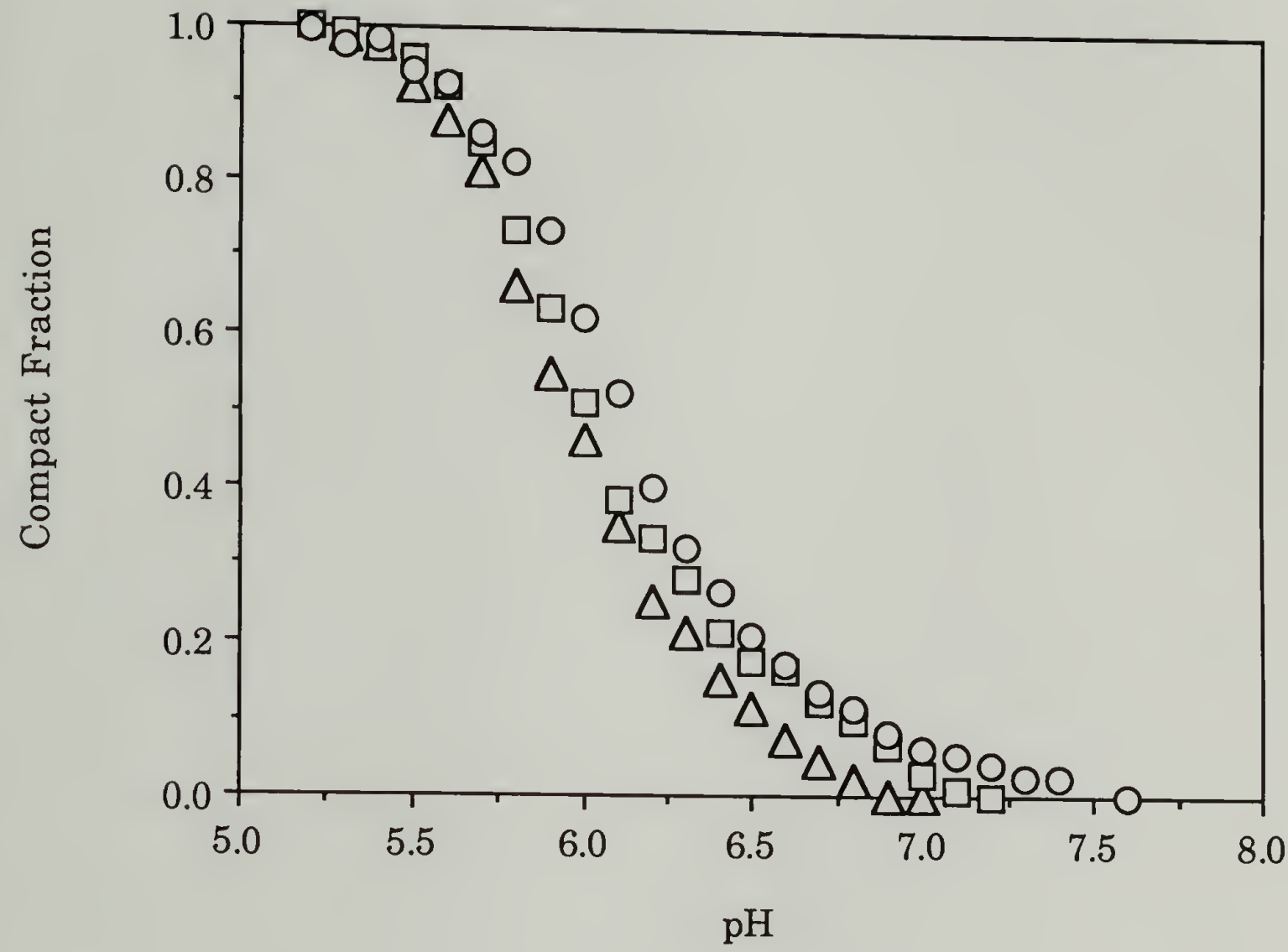


Figure 3.10 Fraction of compact coils of PEAA and EAA-MAA copolymers (1 mg/ml) in aqueous solution (100mM NaCl) as a function of pH at 25 ± 0.2 °C (symbols as in Figure 3.8).

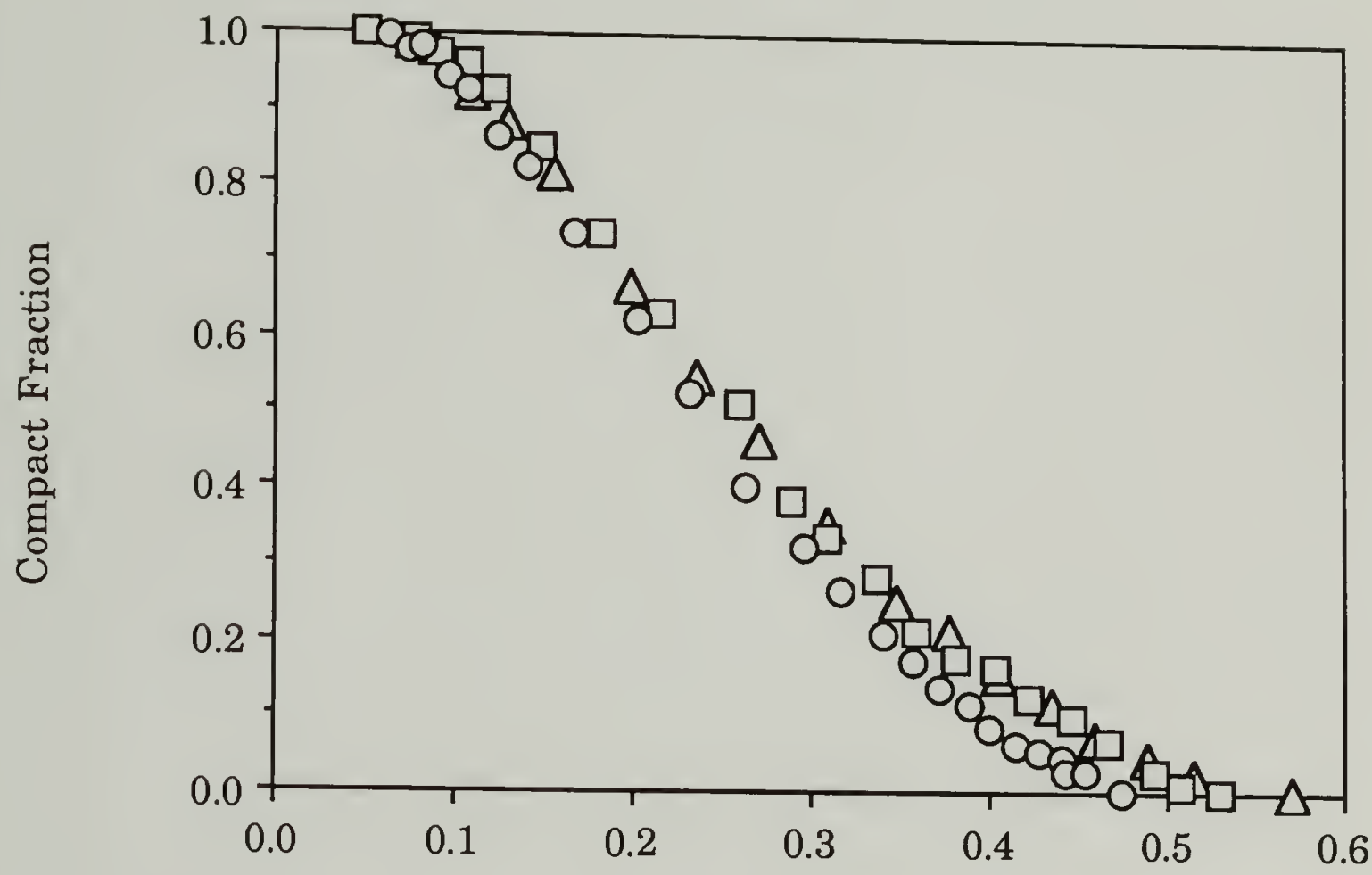


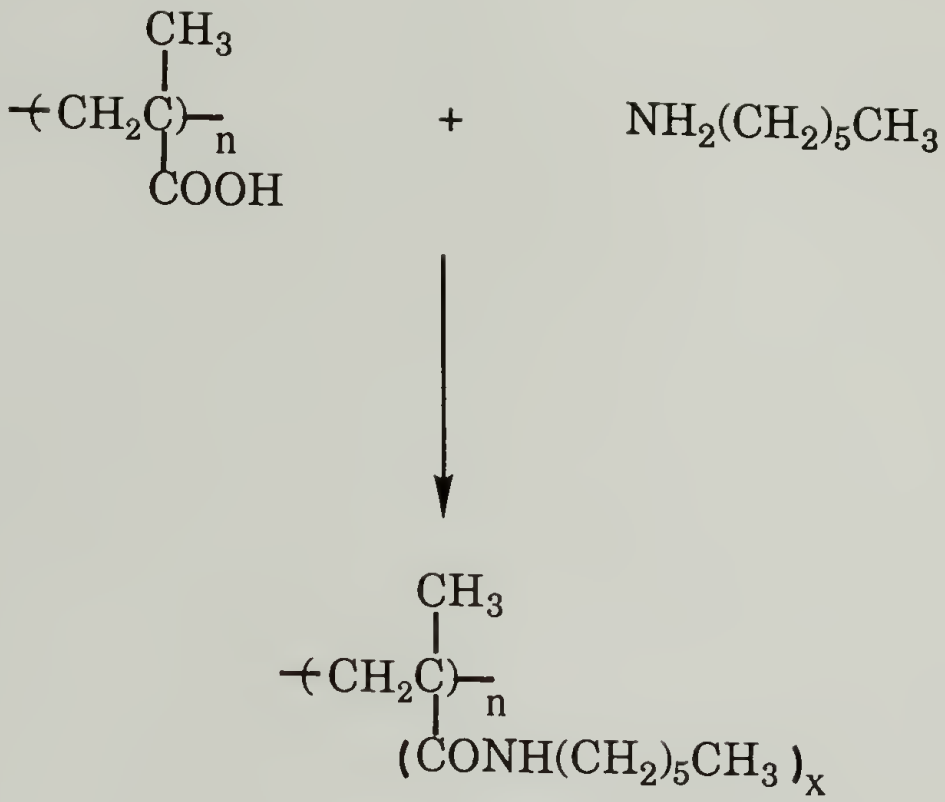
Figure 3.11 Fraction of compact coils of PEAA and EAA-MAA copolymers (1 mg/ml) in aqueous solution (100mM NaCl) as a function of the degree of ionization at 25 ± 0.2 °C (symbols as in Figure 3.8).

quantitative, it is clear that copolymers that did not show the disruption of DPPC vesicles are hydrophobic enough to induce release dye from SUV of EYPC. In the case of copolymer containing 34 % EAA, the quantitative release was observed as pH further reduced to 5.37 although such a behavior was not obtained from copolymer of 19 % EAA. The results explain well the pH-sensitive interaction of copolymers and PEAA with vesicle membranes.

A final note on this result is the slow increase in fluorescence intensity and the pH range where release of dye was observed for PMAA derivative with n-hexyl side chains. In a rather wide range of pH almost the same intensities and shapes of curves were observed. We suggest that because of the intrinsic hydrophobic nature of n-hexyl side chains regardless of pH, the polymer is not so pH-sensitive in interaction with EYPC as PEAA or other copolymers having more cooperative conformational transitions exhibiting very pH-sensitive interaction.

E. Conclusions

The pH-dependent structural reorganization of DPPC vesicle membranes suspended in aqueous buffer solutions of copolymer of 2-ethyl-acrylic acid and methacrylic acid is sensitive to the composition of the copolymer. The shift in critical pH was significant as the EAS content in the copolymer was decreased down to 49 mol%, but with the copolymers of EAA content equal to or less than 40 mol%, there was no clarification of



x = 0.14, mole fraction
based on monomer unit

Figure 3.12 Chemical structure of PMAA modified with n-hexylamine.

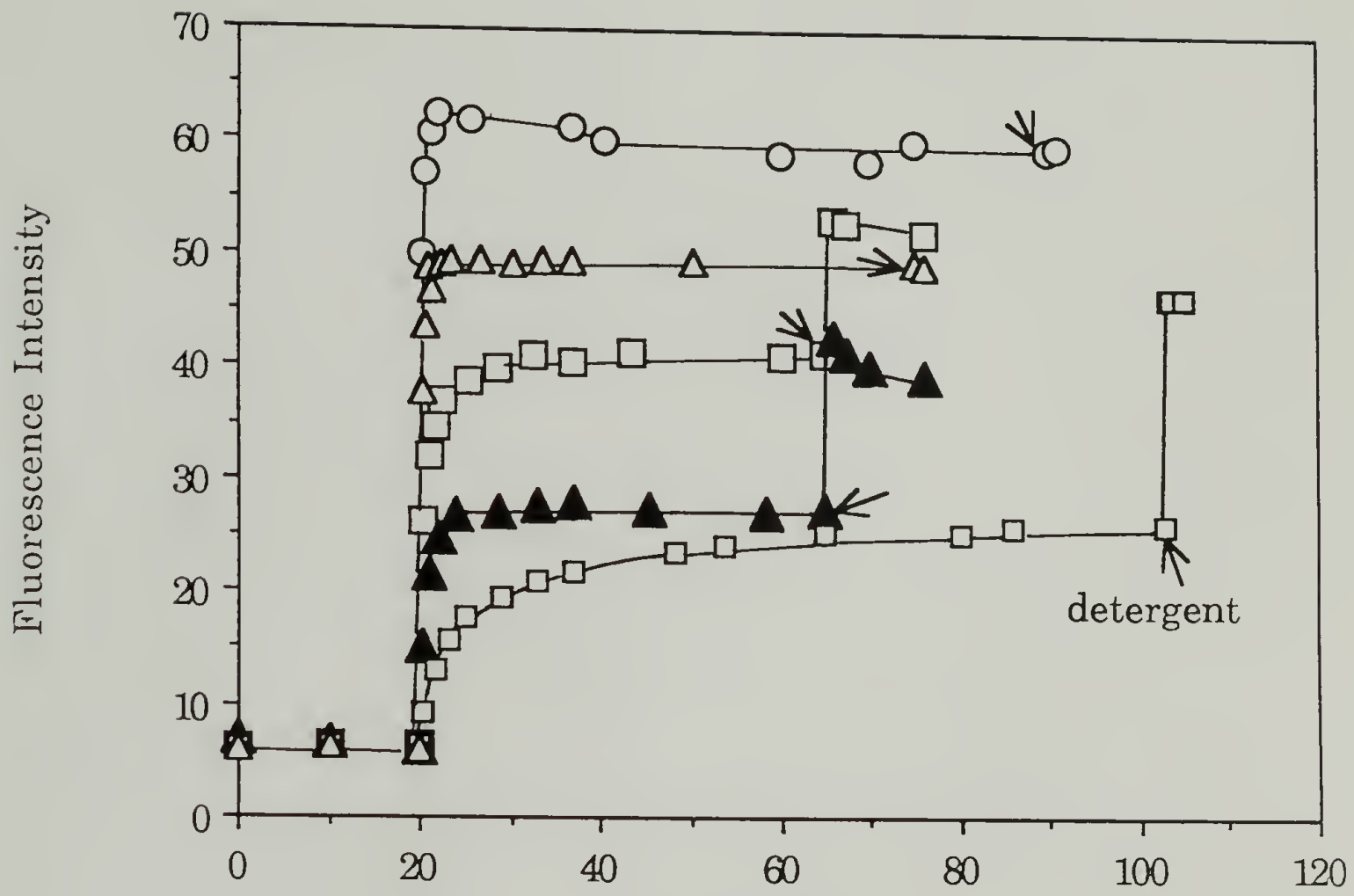


Figure 3.13 Efflux of calcein by polymer (0.2 mg/ml) from sonicated egg yolk phosphatidylcholine (EYPC) (0.04 mg/ ml) vesicles suspended in 10 mM tris-HCl, 100 mM NaCl; (○), PEAA at pH 6.7; (△), 49 % EAA at pH 5.9; (□), 34 % EAA at pH 5.55; (▲), 19 % EAA in copolymer at pH 5.45; (□), PMAA modified with n-hexylamine at pH 5.6 to 5.9. Initial pH was 7.7.

DPPC solution suspensions, and complicated aggregation of DPPC was shown.

The shifts in critical pH for the structural reorganization of DPPC vesicle membranes are found to be attributed to the different hydrophobic interactions of polymers through potentiometric titration of polymers. The ionization at which the transition takes place is shifted to higher values for PEAA compared to the copolymers, owing to stronger hydrophobic interactions in the homopolymer.

Even polymers that do not show disruption of DPPC vesicles induce the release of dye from EYPC SUV membranes. The different hydrophobic interactions of these polymers were again demonstrated by showing complete release of dye for polymers of intermediate hydrophobicity while the partial release of dye was observed for the polymers of weak hydrophobicity. Modified PMAA bearing 14 mol% of n-hexyl side chains exhibited the partial release in a relatively wide range of pH. This seems to be due to the intrinsic hydrophobic nature of the n-hexyl side chains regardless of pH; therefore this polymer is not as pH-sensitive as the other polymers.

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CHAPTER IV

PHOTO-SENSITIVE VESICLE SYSTEMS: USE OF DIARYLIODONIUM SALTS AS PROTON GENERATORS

A. Abstract

Photosensitive 3,3'-dicarboxydiphenyliodonium bisulfate (HSO_4^-), hexafluorophosphate (PF_6^-), and iodide (I^-) were prepared. Strong acids (H_2SO_4, HPF_6 and HI) were produced upon irradiation of aqueous solutions of the iodonium salts at 254 nm, and large changes in solution pH were observed. Structural reorganization of phosphatidylcholine vesicle membranes by poly(2-ethylacrylic acid) (PEAA) was successfully demonstrated upon irradiation of iodonium salt solutions. Reorganization of vesicle membrane structure was detected by monitoring the efflux of entrapped calcein from egg yolk phosphatidylcholine (EYPC) membranes by PEAA in the presence of the diaryliodonium salts. PEAA induced quantitative release of dye upon irradiation while the diaryliodonium salt itself had no effect on release of dye.

B. Introduction

The pH-dependent conformational transition of poly (2-ethylacrylic acid) (PEAA) has been described in Chapter III for application in sensitization of synthetic bilayer membranes to changes in pH (1), temperature (2), light intensity (3) and solute concentration (4). The mechanism of the membrane response has been shown to consist of a structural reorganization of the membrane lipid from a vesicular form at high pH to a mixed polymer-lipid micelle at low pH, the reorganization being driven by collapse of the polymer chain from an extended, hydrophilic form to a compact, hydrophobic coil upon acidification (5). As variations to adjust either the critical pH for membrane reorganization or the cooperativity of the structural transition, in our previous work the effects of the tacticities (1) and molecular weights of PEAA (6), and of compositions of copolymers of EAA and MAA on the structural reorganization of the lipid membranes were studied (see Chapter III)

Since the iodonium salts were originally developed by Hatman and coworkers (7, 8) Beringer et al.(9, 10) and other groups (11-16) have synthesized various kinds of iodonium salts and investigated the synthetic procedures and physical properties. After the fact of their photosensitivity was recognized, detailed investigations of the photolysis of certain halides (17, 18) and hydroxides (19, 20) were made. In addition, apart from a few diaryliodonium fluoroborate salts which are reported in the literature, diaryliodonium salts possessing other complex metal halide anions did not appear to have been prepared.

It was not until Crivello et al.(21-27) and others (28, 29) discovered diaryiodonium salts with complex metal halide counterions that attention was given to them as initiators in photopolymerizations. Such compounds are now known to be very efficient photoinitiators for polymerization of a variety of cationically polymerizable monomers. Useful features of these photoinitiators include high photosensitivity, excellent dark stability and ease of isolation (in contrast to dialkyliodonium salts). It has been found that solutions of these photoinitiators in the most reactive multifunctional monomers may be stored in the dark for several years without gelation.

Early in the study of mechanism of the photolysis of diaryliodonuim salts, the reaction was considered to be a redox process in which the diaryliodonium salt is reduced and the solvent oxidized (26). Such a reaction generates strong protonic acids. As described previously it would be of interest to be able to sensitize vesicle membranes to other stimuli. Irradiation as a tool of sensitization has been reported (3, 30). Using the iodonium salts as protonic acid sources would be another method for sensitization of vesicle membranes by polymers which was described in CHAPTER III. Therefore, we describe herein the synthesis of iodonium salts which are decomposed upon irradiation and produce strong protonic acids. The iodonium salt was employed as a proton source to decrease pH in the structural reorganization of phophatidylcholine vesicle membranes by PEAA and to effect release of dye from EYPC SUV.

C. Experimental

1. Materials

All reagents and their sources are listed below. The reagents were used as received unless otherwise stated.

Acetic anhydride (A)

Acetone, A.C.S. grade (F)

Benzoic acid (A)

Calcein (A)

L-α-Dilauroyl phosphatidylcholine (DLPC), 99 % (S)

L-α-Dimirystroyl phosphatidylcholine (DMPC), 99 % (S)

Dimethylsulfoxide-d_6 (A)

Egg yolk phosphatidylcholine (EYPC) (S)

Hydrochloric acid, 0.100 ± 0.001 N volumetric standard solution (F)

Iodine, A.C.S. reagent (A)

3-Iodobenzoic acid (A)

Methanol, A.C.S. grade (F)

Potassium hexafluorophosphate (A)

Potassium iodate, A.C.S. reagent (A)

Sepharose CL 4B-300 (S)

Sodium hydroxide, 0.100 ± 0.001 N volumetric standard solution (F)

Sodium azide (F)

Tris(hydroxymethyl)aminomethane (Tris), A.C.S reagent (A)

Triton X-100, scintillation grade (Am)

Sources

(A) Aldrich Chemical Co. (Milwaukee, WI)

(Am) Amersham Corporation (Alrington Heights, IL)

(F) Fisher Scientific (Boston, MA)

(S) Sigma Chemical Co. (St. Louis, MO)

2. Preparation of iodonium salts

a. Synthesis of 3,3'-dicarboxydiphenyliodonium salts (bisulfate, hexafluorophosphate, and iodide). To a suspension of iodyl sulfate prepared by vigorous stirring of a mixture of 1.925 g (7.6 mmol) of iodine, 5.0 g (23.2 mmol) of potassium iodate and 20 ml of sulfuric acid at room temperature for 3 hours, there were added 0.5 ml of acetic anhydride and 9.5 g (78 mmol) of benzoic acid while maintaining the temperature below 10 ^{o}C. After stirring for 8 hours in an ice-bath and for 15 hours at room temperature (negative starch-iodide test), the mixture was diluted with 100 ml of cold water. An ether-soluble precipitate was removed by filtration and the filtrate was extracted with 150 ml of ether four times. A small amount of ether in the aqueous phase was removed by evaporation and the final volume was measured as 120 ml. The solution was divided into three 40 ml volumes. One portion was kept in the refrigerator for 24 hours, and further reactions were carried out with the other two portions. After 24 hours in the refrigerator, the precipitated 3,3'-dicarboxydiphenyliodonium bisulfate was filtered and washed with water, acetone, and ether. The product was dried under vacuum at room temperature in the dark for more than 48 hours. To the second 40 ml aliquot there were added 4.3 g of

potassium iodide and a few mg of sodium bisulfite in 8.4 ml water. Immediately a yellow precipitate formed, was collected by filtration, washed with water and with ether and dried in vacuo to give 1.9 g (30 %) of 3,3'-dicarboxydiphenyliodonium iodide. The final 20 ml of bisulfate solution was reacted with 1.0 g of potassium hexafluorophosphate in 100 ml of water at room temperature. The initially homogeneous mixture was stirred for 3 hours and the resulting precipitate was collected, washed with water and ether, and dissolved in methanol and kept in the refrigerator. After 24 hours 3, 3'-dicarboxydiphenyliodonium hexafluorophosphate was filtered, washed with methanol and ether, and dried under vacuum to give 0.54 g (20 %) of product.

b. Measurements. The melting points of the three iodonium salts were measured with Fisher-Johns Melting Point Apparatus (Fisher Scientific). UV spectra of the iodonium salts and of the mixtures of iodobenzene and 3-iodobenzoic acid in water were obtained on a Beckman DU-7 UV/VIS spectrometer. ^{1}H NMR spectra were recorded on a Varian XL 200 spectrometer with dimethylsulfoxide-d_6 (DMSO-d_6) as solvent.

3. Structural reorganization of DLPC and DMPC vesicles using iodonium salts

a. Preparation of vesicle samples. A solution of 15 mg DMPC in 3 ml $CHCl_3$ was evaporated in a long-neck round-bottomed flask to dryness on a rotary evaporator. After drying under vacuum for more than 6 hours the DMPC film was hydrated with 15 ml of 10mM Tris buffer (50 mM NaCl and

0.01 % NaN_3) at 35-40 °C and vortexed for 5 to 10 minutes while maintaining temperature above 35 °C.

b. Measurements. Rates of pH depression of aqueous solutions of iodonium salts (4 mM) in the absence and in the presence of PEAA (1 mg / 1.4 ml) were measured in quartz cells of 0.4 cm inner diameter upon irradiation at 30°C in a Rayonet minireactor (Southern New England Ultraviolet Co.) equipped with a merry-go-round sample holder and four 254 nm RPR mercury lamps. The sample cells were periodically withdrawn and shielded from the light and pH was measured immediately.

For measurement of optical densities, solutions of PEAA and 3,3'-dicarboxydiphenyliodonium hexafluorophosphate and lipid in 10 mM Tris buffer were mixed so that the final concentrations of PEAA and lipid were equal by weight, and that of iodonium salt was 4 mM. The mixtures were then irradiated as described above. The sample cells were periodically withdrawn and shielded from the light, and kept in a 30 °C bath to give a total of 30 minutes incubation time. Optical density at 600 nm and pH were then measured at 25 ±0.2 °C.

4. Kinetics of structural reorganization of EYPC MLV

a. Preparation of vesicle samples. EYPC MLV (1 mg / ml) was prepared as described in section 2-a of this Chapter. Vortexing was carried out for 10 minutes at room temperature.

b. Measurements. For optical density measurement, vesicle suspensions (2 mg / ml) and PEAA solutions (2 mg / ml) were combined in equal volumes. Optical density was measured on a Beckmann DU-7 UV/VIS spectrometer as a function of time. After measurement , the sample was shaken to check if the precipitation of the lipid during the measurement caused any decrease in optical density.

5. Release of calcein from EYPC SUV membranes by PEAA using iodonium salt

a. Preparation of vesicle samples. EYPC small vesicles containing calcein were prepared by the method described in Experimental 4.b of Chapter III. The concentration of calcein was 250 mM in 1mM Tris buffer containing 100 mM NaCl. The concentration of the lipid was also determined as in Experimental 4.a of Chapter III and 0.038 mg / ml was used in measurements.

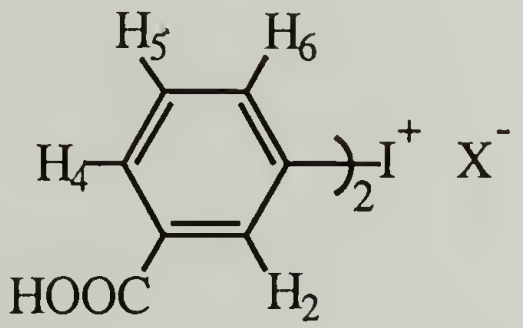
b. Measurements. The proper concentrations of PEAA, lipid (0.2 mg and 0.038 mg/ml, respectively) and 3,3'-dicarboxydiphenyliodonium bisulfate(1.6 mM) were chosen in order to shorten the irradiation time for release of dye. A 2 ml aliquot of sample was transferred to a quartz cuvet and release of dye was triggered upon irradiation at 254 nm with a monochromatic Pen-Ray lamp (UVP, 4500 mw / cm^2 at 2.5 cm) through the cuvet window. After irradiation, the fluorescence intensity from the sample was immediately recorded on a Perkin-Elmer MPF-66 fluorescence

spectrometer and pH was measured at 25 ± 0.2 °C. Maximum fluorescence intensity was obtained by adding 0.05 ml of 15 % Triton X-100 solution.

D. Results and Discussion

3, 3'-Dicarboxydiphenyliodonium salts with bisulfate, hexafluorophosphate, and iodide counterions were obtained as pale - yellow powders. Melting temperatures were determined to be 162-164 °C and 173-178 °C for the bisulfate and hexafluorophosphate salts, respectively. In the case of the iodide, a first melting was observed at about 121 °C with a change from pale - yellow powder to white crystals; the white crystals then melted at 183-185 °C. This result was the same as that reported (9), and is attributed to the decomposition of the iodonium salt into 3-iodobenzoic acid followed by melting of the acid.

^{1}H NMR spectra of the iodonium bisulfate and hexafluorophosphate salts are shown in Figures 4.1 and 4.2, and that of 3-iodobenzoic acid under the same conditions is compared in Figure 4.3. The structure of the iodonium salt is



X=HSO_4^-, PF_6^-

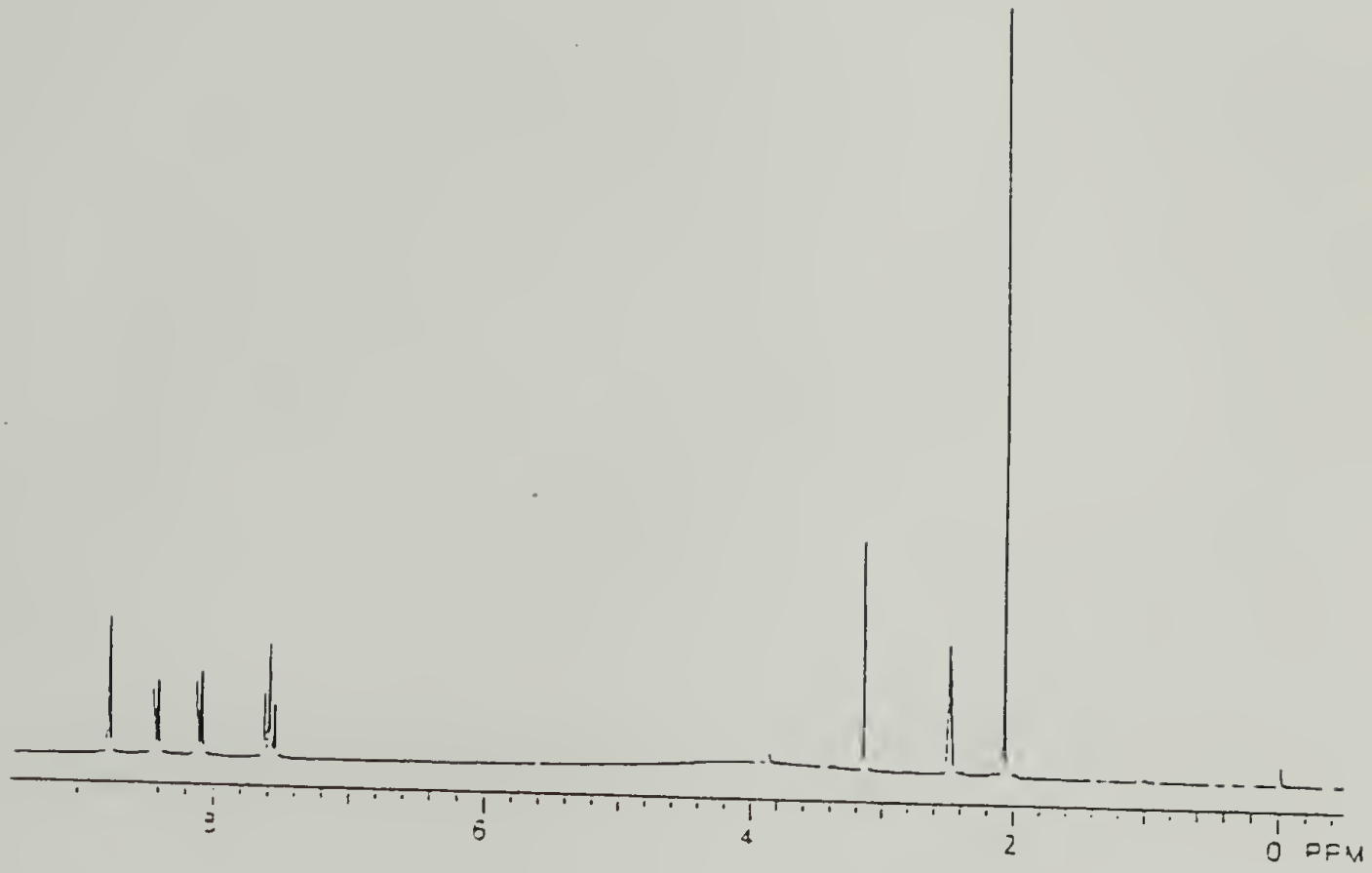


Figure 4.1 ^{1}H NMR spectrum of 3,3'-dicarboxydiphenyliodonium bisulfate in DMSO-d_6 at 23 °C.

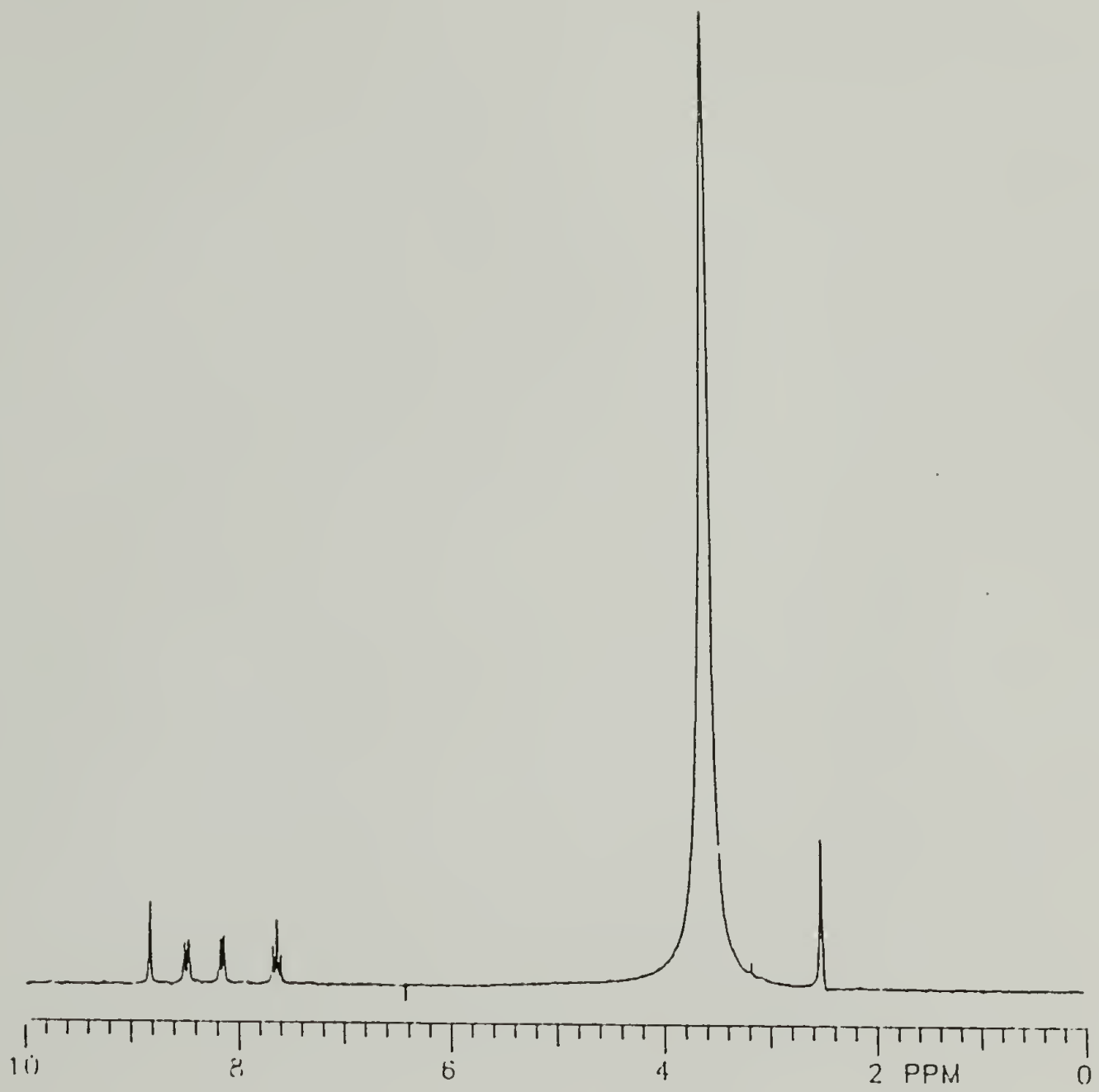


Figure 4.2 ^{1}H NMR spectrum of 3,3'-dicarboxydiphenyliodonium hexafluorophosphate in DMSO-d_6 at 23 °C.

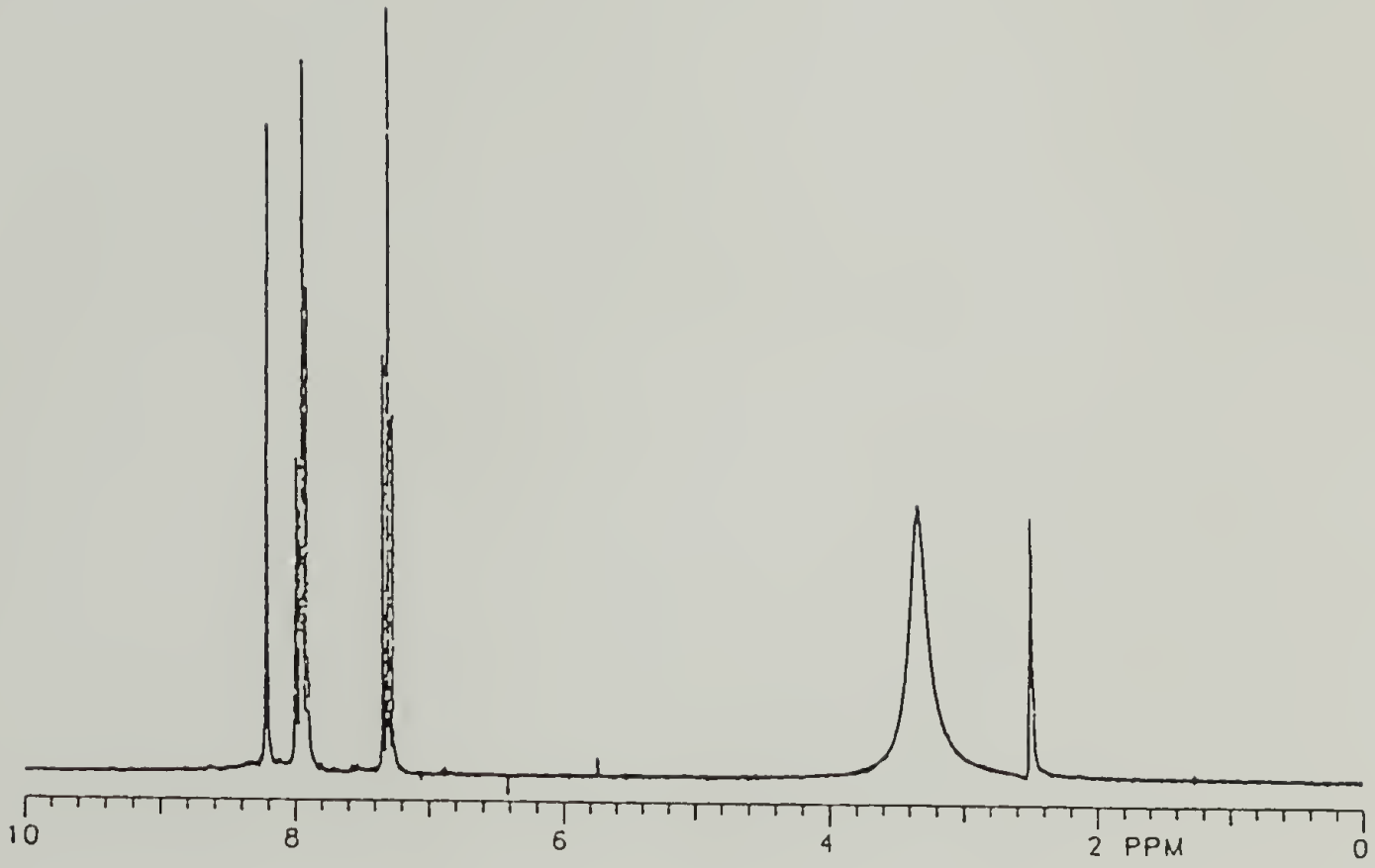


Figure 4.3 ^{1}H NMR spectrum of 3-iodobenzoic acid in DMSO-d_6 at 23 °C.

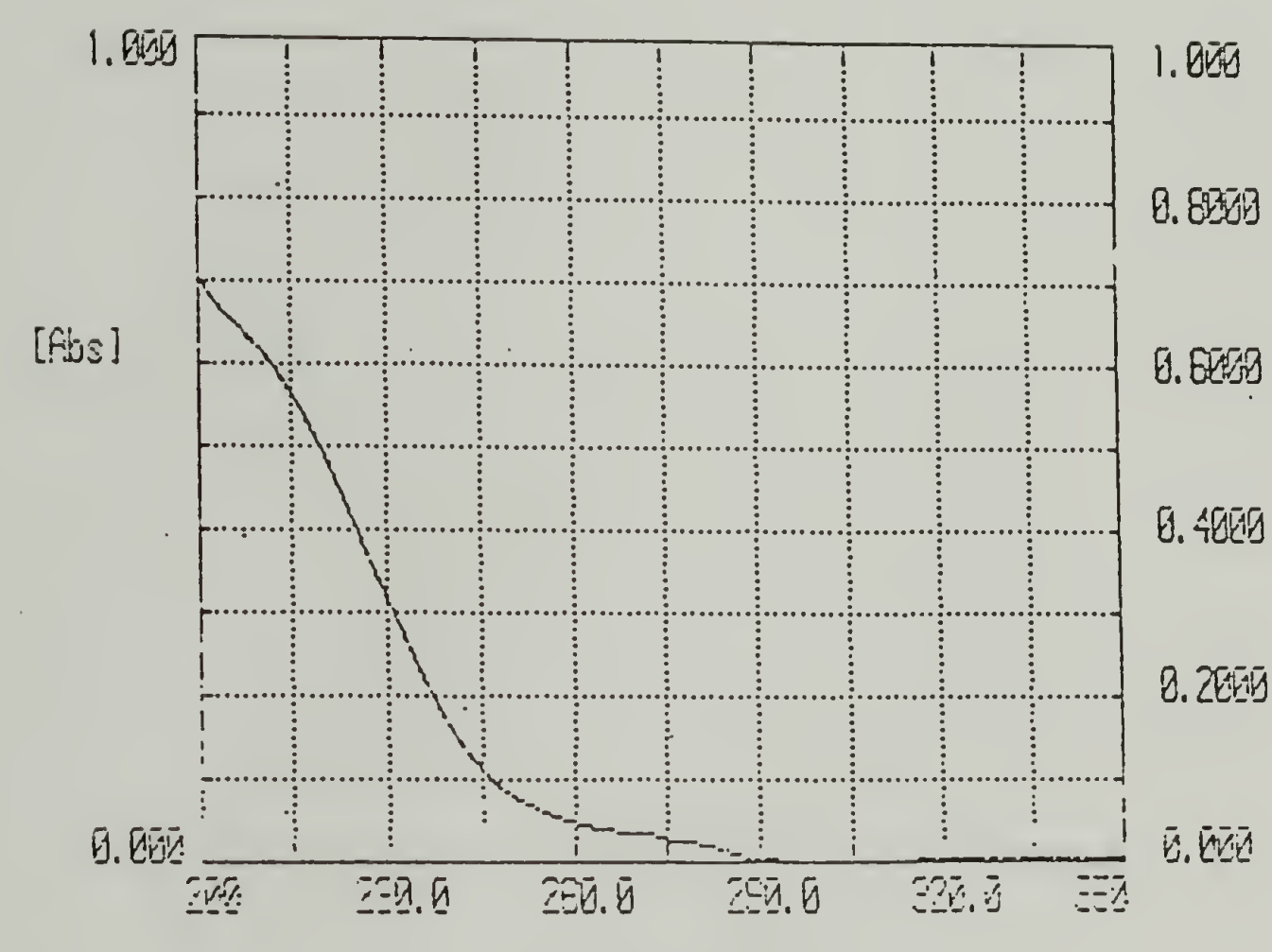


Figure 4.4 Ultraviolet spectra of 3,3'-dicarboxydiphenyliodonium bisulfate and hexafluorophosphate (they are overlapped) in aqueous solution.

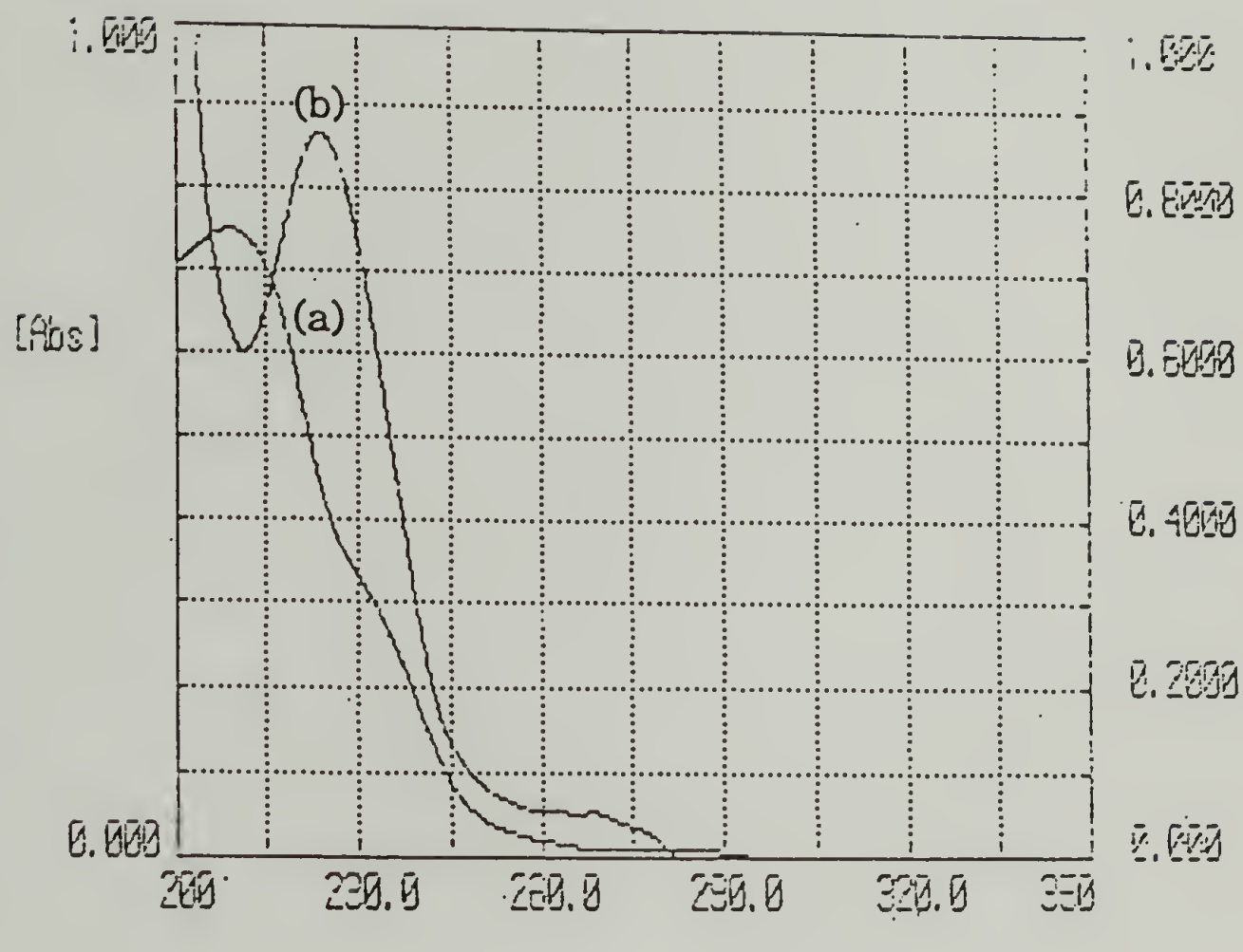


Figure 4.5 Ultraviolet spectra of (a) benzoic acid and (b) 3-iodobenzoic acid in aqueous solution.

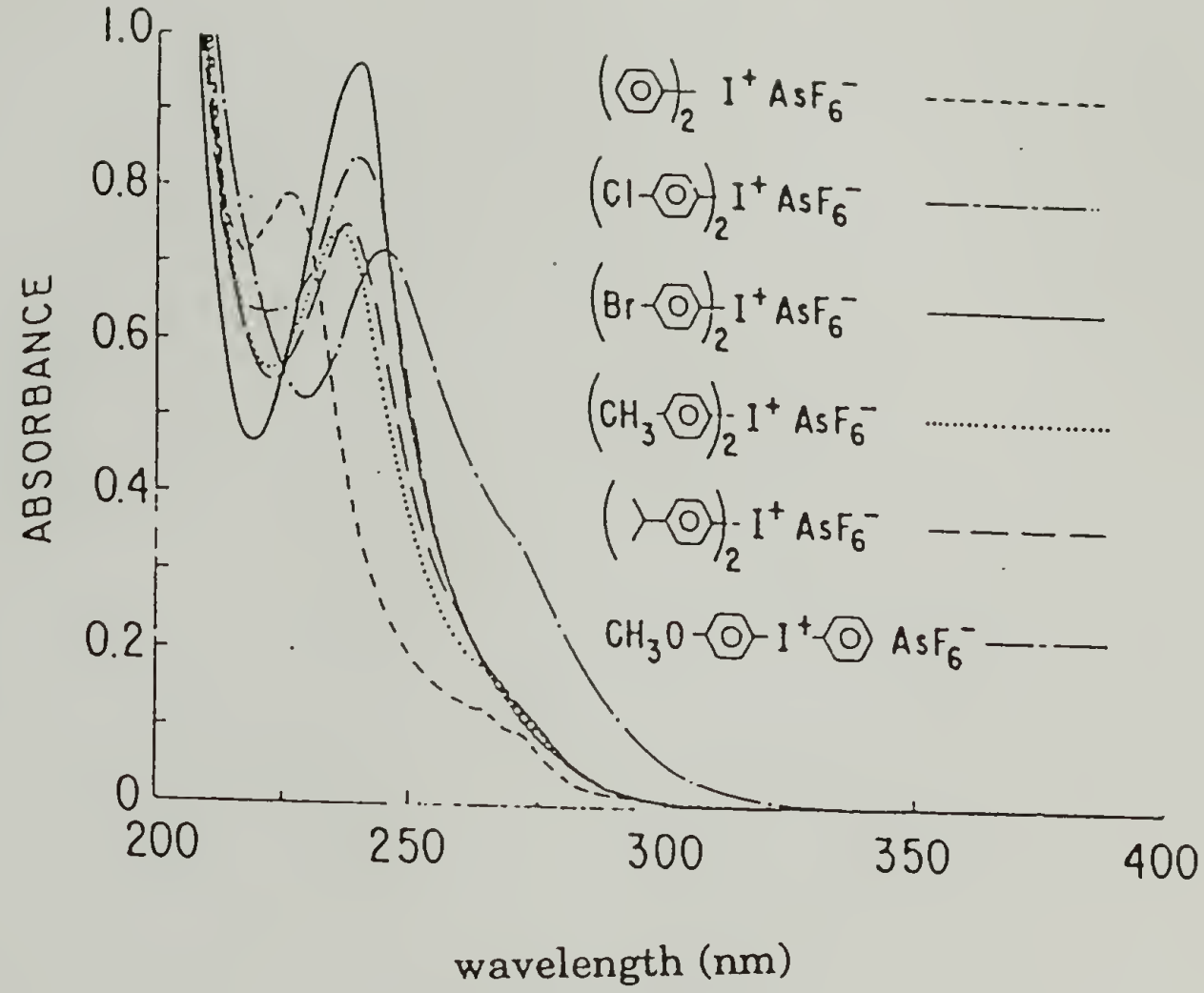


Figure 4.6 Ultraviolet spectra of substituted diaryliodonium salts. From Crivello, J. V. and Lam, J. H. W., Macromolecules, 10, 1307, 1977.

The apparent triplet at 7.59 ppm is assigned to H_5 and is actually a combination of two doublets arising from splitting by H_4 and H_6. The doublets at 8.1 and 8.42 ppm are assigned to H_4 and H_6, respectively, and H_2 appears as a singlet at 8.77 ppm. The peaks at 2.43 in both spectra are from solvent (DMSO-d_6) and between 3 to 5 ppm water peaks are shown. In the spectrum in Figure 4.1, acetic anhydride is detected at 2.05 ppm. These spectra show that 3-iodobenzoic acid, which was a reaction intermediate, was removed completely from the iodonium salts. The counter ion did not affect the ^1H NMR spectra, as reported previously (22). UV spectra of the bisulfate and hexafluorophosphate salts, benzoic acid and 3-iodobenzoic acid are shown in Figures 4.4 and 4.5. Most diaryliodonium salts are known to have λ_{max} in the range of 225 to 250 nm (22) as shown in Figure 4.6, but the 3,3'-dicarboxydiphenyliodnoium salts prepared here did not show λ_{max} and absorbance increases gradually at shorter wavelength. There was no difference in the UV spectra for the two iodonium salts.

A general mechanism for the photolysis of diaryliodonium salts was proposed by Crivello and Lam (22), and was similar to that reported earlier by McEwen and coworkers (19). The mechanisms are as follows.

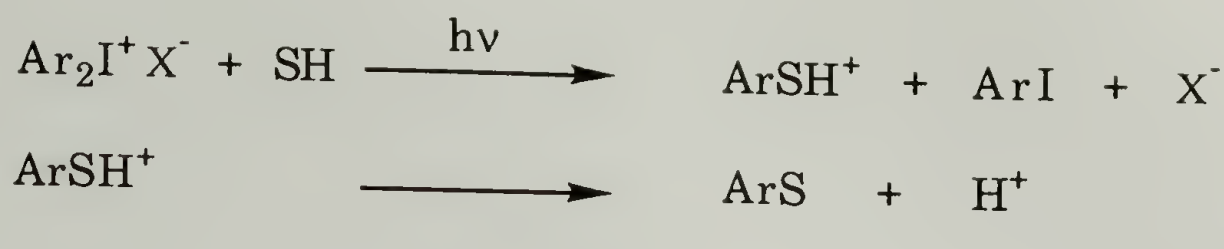
Major

$$Ar_2I^+X^- \xrightarrow{h\nu} ArI^+\cdot + Ar\cdot + X^-$$

$$ArI^+\cdot + SH \longrightarrow ArI^+{-}H + S\cdot$$

$$ArI^+{-}H \longrightarrow ArI + H^+$$

Minor



The major pathway involves the facile decomposition of the excited iodonium salt to an aryliodo radical-cation, an aryl radical and an anion. This process should be highly efficient due to the very low bond energy of the C-I bond (26-27 kcal/mol) (31). The fate of the aryl radicals generated in the second step in the major scheme is to give rise to biphenyl products via dimerization and to benzoic acid via hydrogen abstraction from the solvent S-H. Using deuterated solvents, the hydrogen abstraction mechanism was confirmed (22). Interaction of the aryliodo radical-cation with the solvent (S-H). generates a protonated iodoaromatic compound which rapidly deprotonates, producing the acid species, H^+X^-. This acid was exploited to decrease the pH in our system.

Figures 4.7 and 4.8 compare the UV spectra of the photolysis products from 3, 3'-dicarboxydiphenyliodonium hexafluorophosphate with the spectra of mixtures of 3-iodobenzoic acid and benzoic acid. In the initial stages of photolysis the UV spectra of the photolysis products are similar to the spectrum of an equimolar mixture of benzene and 3-iodobenzoic acid (compare spectra 1 and 2 of Figure 4.7 to spectrum 3 of Figure 4.8). However, as the photolysis time increases the spectrum becomes similar to that of a mixture containing mainly benzoic acid (compare spectrum 5 of Figure 4.7 to spectrum 5 of Figure 4.8). This result is explained by a secondary photolysis mechanism. From the primary photolysis in the

initial stage, equal amounts of benzene and 3-iodobenzoic acid are produced , but as photolysis proceeds further the weak C-I bond in 3-iodobenzoic acid is cleaved and additional benzoic acid is produced. The other product from the photolysis of the iodonium salt may be hydriodic acid, HI and a change in color of the solution into yellow or brown is evidence for the presence of HI. Thus, it is concluded that the results here seem to follow the photolysis mechanism reported by others.

The structural reorganizations of DMPC and DLPC were studied by measuring optical density at 600 nm in the presence of PEAA and 3,3'-dicarboxydiphenyliodonium hexafluorophosphate. Since optical density changes as a function of time above the melting transition temperature of lipid after changing pH, all measurements were conducted after the same time period at the same temperature. The results are summarized in Figure 4.9, which shows the typical curve of optical density as a function of pH, as published previously in our laboratory (1-4). Complete disruption of the vesicles (optical density measured as zero) was found at pH 6.6 and 6.7 for DMPC and DLPC, respectively. The difference in the critical pH is attributed to the fact that DMPC is more stable than DLPC, owing to its increased acyl chain length. The pH changes occurring during optical density measurements are shown in Figure 4.10 as a function of irradiation time, and were consistent for DMPC and DLPC. The pH drops almost linearly with irradiation time in the absence of polymer which is similar to the result reported (as conversion versus irradiation time) (21). In the presence of polymer the pH drop is slower and reflects the buffer capacity of polymer in the solution.

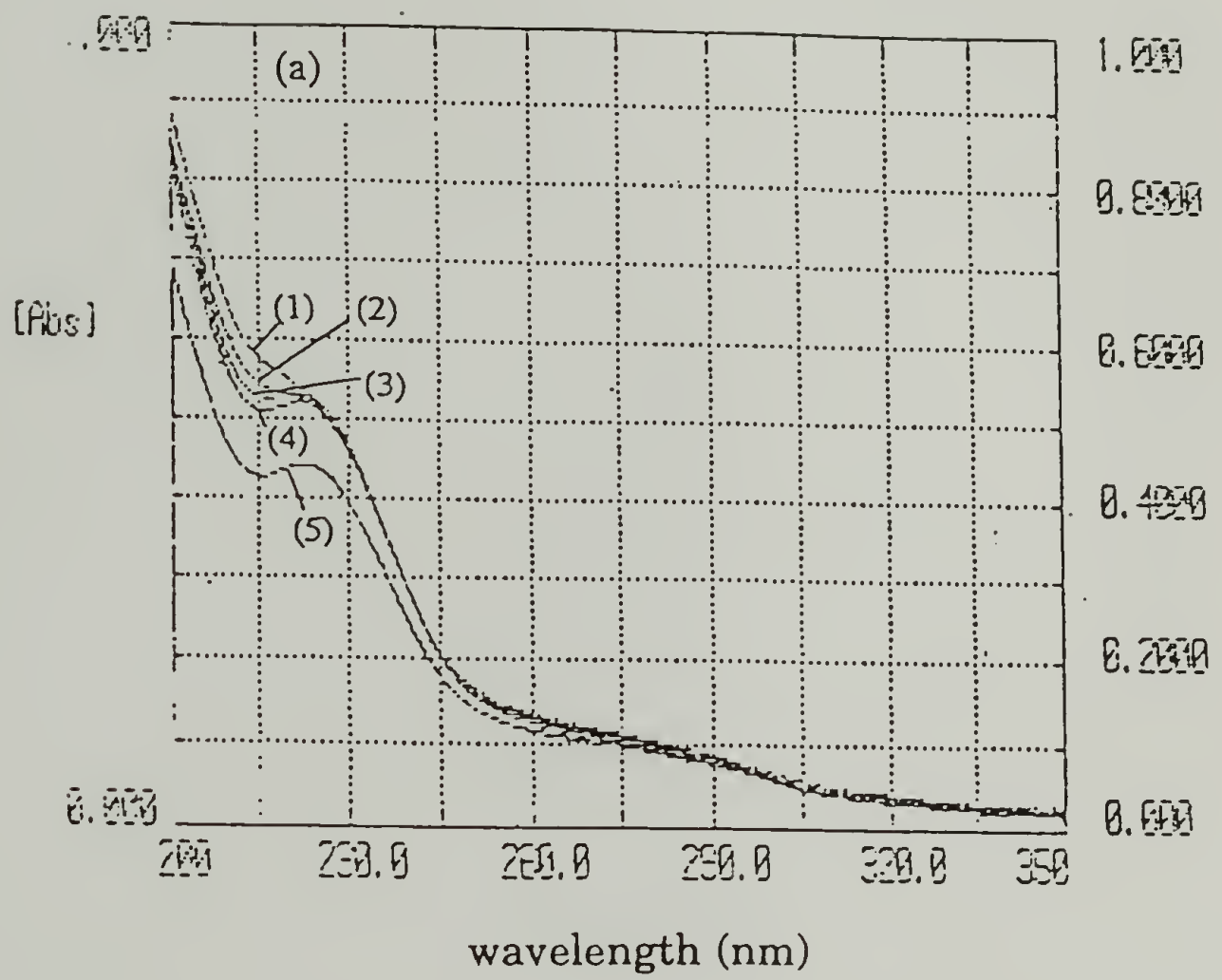


Figure 4.7 Ultraviolet spectra of 1.3 mM of 3,3'-dicarboxydiphenyliodonium hexafluorophosphate in aqueous solutions after the photolyses for different time periods (minutes); (1) 5, (2) 10, (3) 15, (4) 20, (5) 25.

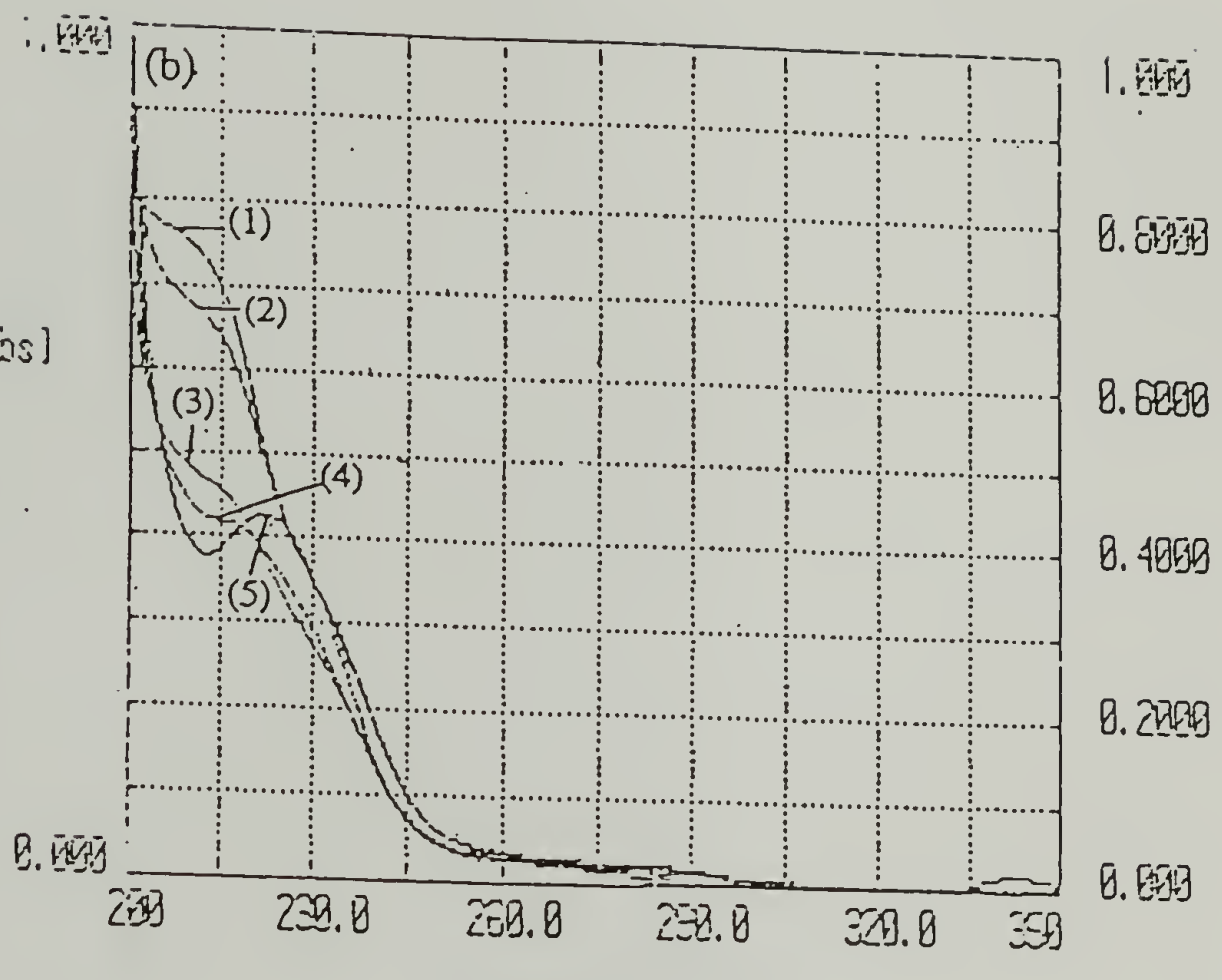


Figure 4.8 Ultraviolet spectra of mixtures of benzoic acid and 3-iodobenzoic acid in aqueous solutions; (1) 1:9, (2) 3:7, (3) 5:5, (4) 7:3, (5) 9:1 mole ratios of benzoic acid/3-iodobenzoic acid.

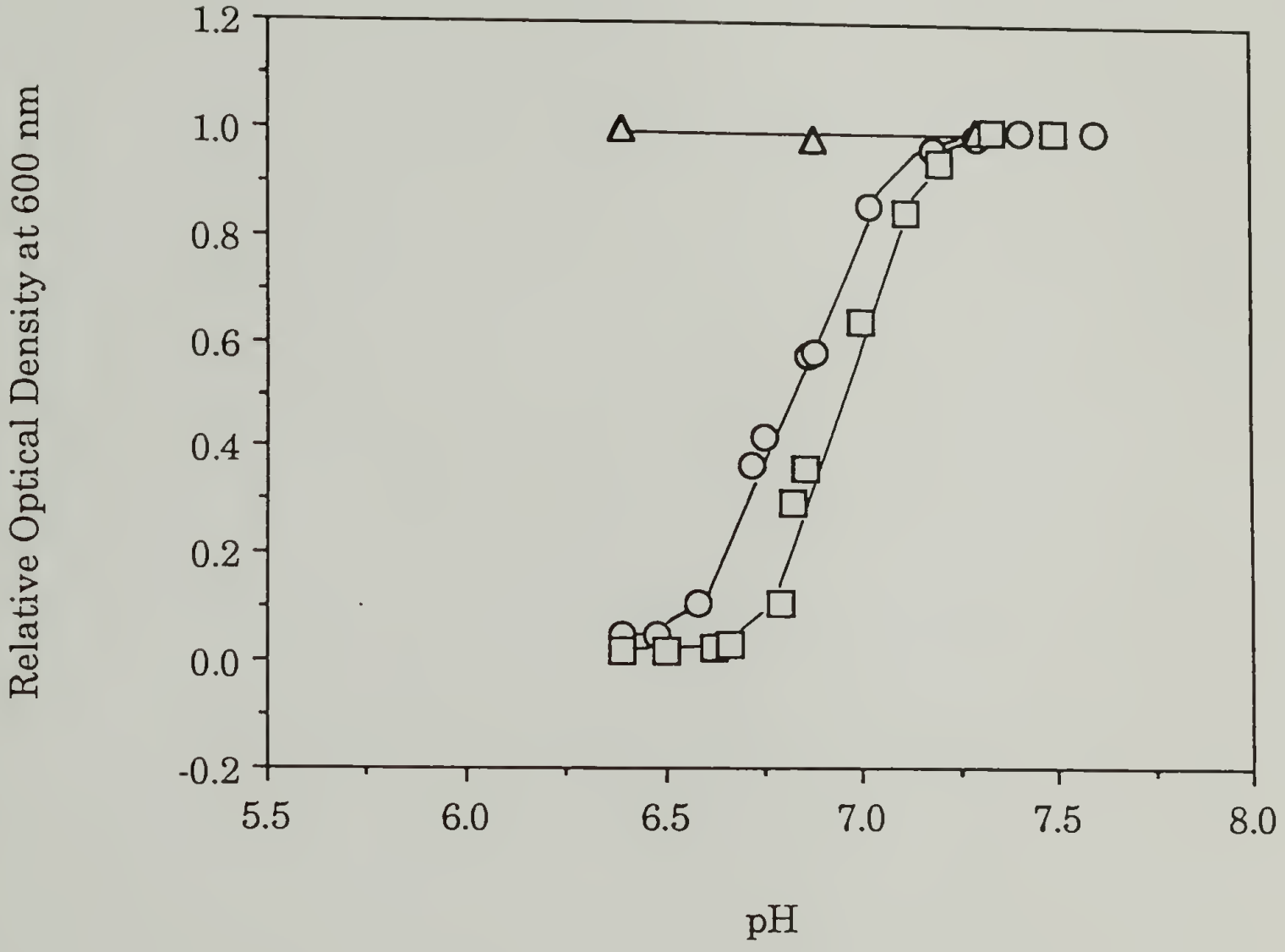


Figure 4.9 Optical density (relative to that before photolysis) at 600 nm as a function of pH during the photolyses of 1:1 lipid/polymer mixtures in the presence of 4 mM of 3,3'-dicarboxydiphenyliodonium hexafluorophosphate in 10 mM tris buffer; (○) DMPC at 30 °C, (□) DLPC at 24 °C, (Δ) polymer-free control. Estimated errors are about ± 0.02 in pH and in relative optical density.

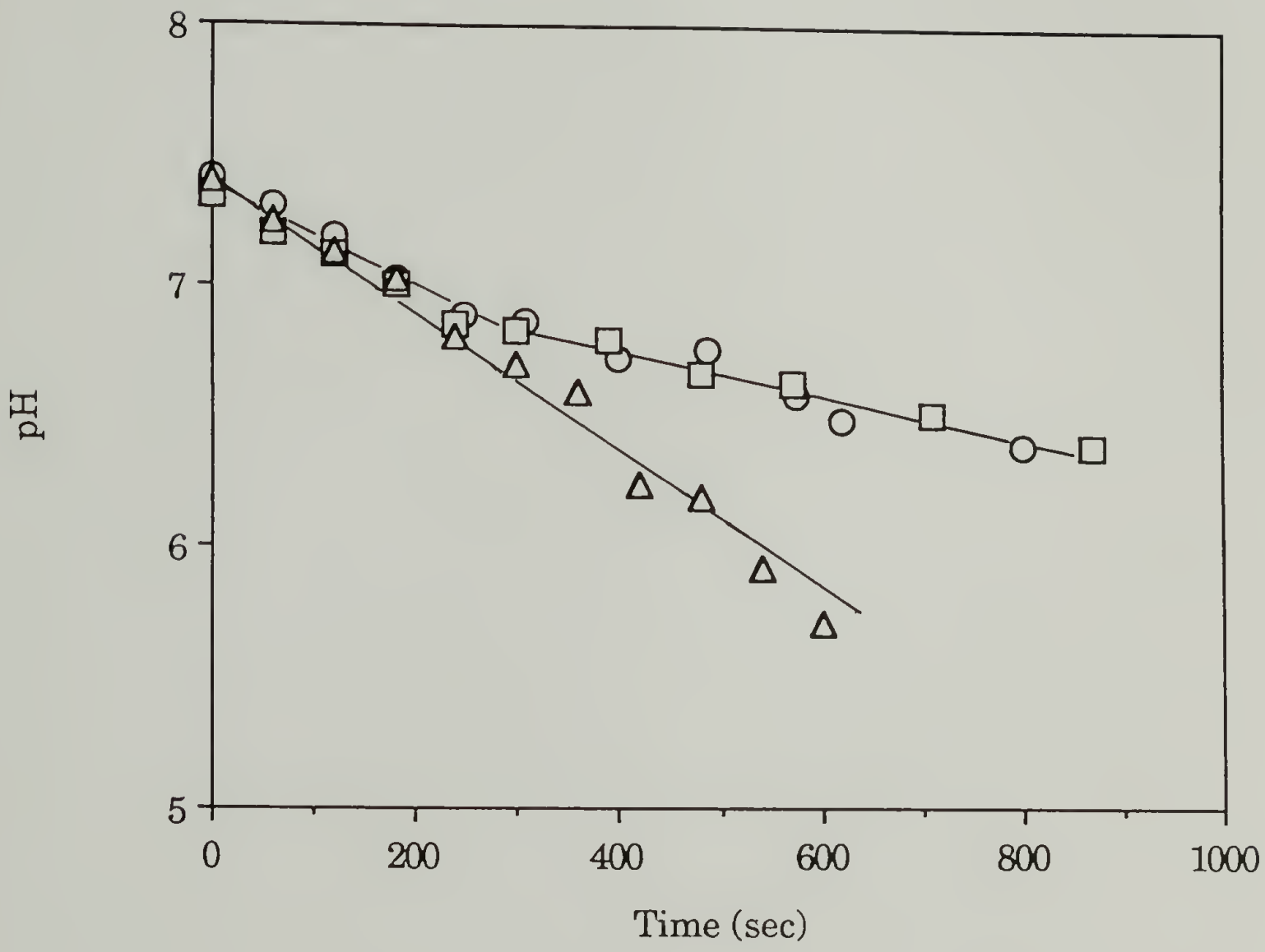


Figure 4.10 pH as a function of irradiation time from samples of 1:1 lipid/polymer mixtures in the presence of 4 mM of 3,3'-dicarboxydiphenyliodonium hexafluorophosphate in 10 mM tris buffer (symbols as in Figure 4.9).

Release of dye from EYPC SUV membranes by PEAA has been demonstrated by Seki and Tirrell (2). Quantitative release was observed. In the present work dicarboxydiphenyl iodonium hexafluorophosphate was applied in sensitization of EYPC membranes for release of dye. In order to get preliminary information on the structural reorganization behavior of EYPC vesicle membranes, optical density measurement as a function of time at different pH was carried out by adding HCl solution. The results are summarized in Figure 4.11. At high pH little reduction in optical density was observed over 30 minutes and most of the decrease occurred in a short time i.e., within five minutes. When the pH was reduced to below 6.4 a drastic drop in optical density was observed and even after 30 minutes optical density still decreased. Clarification of EYPC MLV membranes was almost complete after three hours at pH 6.25.

The results of dye-release by PEAA in the presence of 3,3'-dicarboxydiphenyliodonium bisulfate upon irradiation at 254 nm are summarized in Figures 4.12 to 4.14. In the control experiment, i.e. in the absence of polymer, it was found that the iodonium salt itself could not induce the release of dye and by adding detergent quantitative release was obtained as shown in Figure 4. 12. When PEAA was employed in this system, upon irradiation immediate release of dye was observed as demonstrated previously in Tirrell's group (3). By controlling irradiation time period, i.e., pH complete and partial release were obtained and the results are shown in Figure 4.13. Therefore, 3, 3'-dicarboxydiphenyliodonium salts were very useful as a proton-generating source and structural reorganization of DMPC and DLPC vesicles and

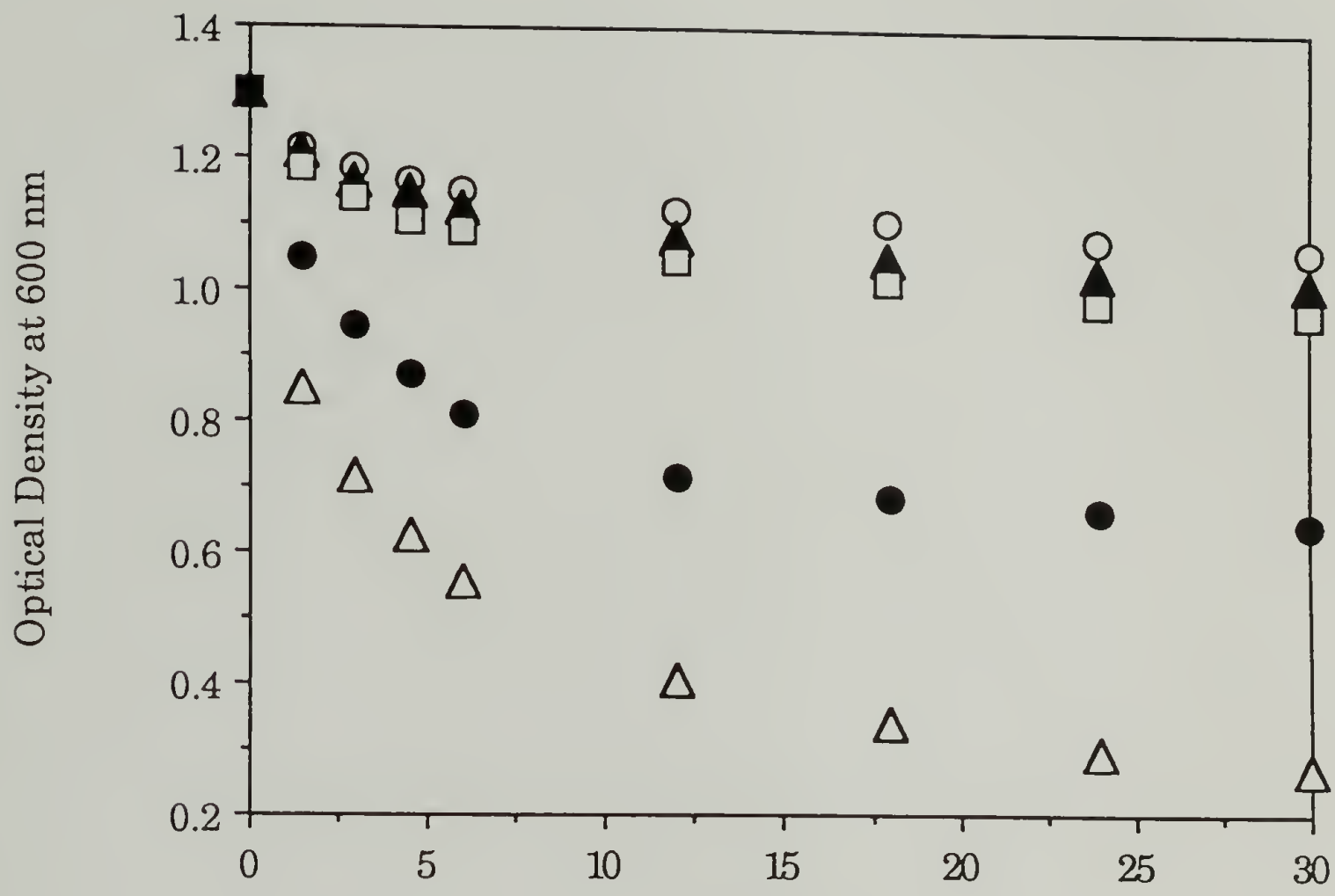


Figure 4.11 Optical density of the mixtures of EYPC (MLV) and PEAA (1 mg/ml each) in 1 mM tris buffer (100 mM NaCl) as a function of time at different pH; (○) pH 6.69, (▲) pH 6.61, (□) pH 6.59, (●) pH 6.37, (△) pH 6.26.

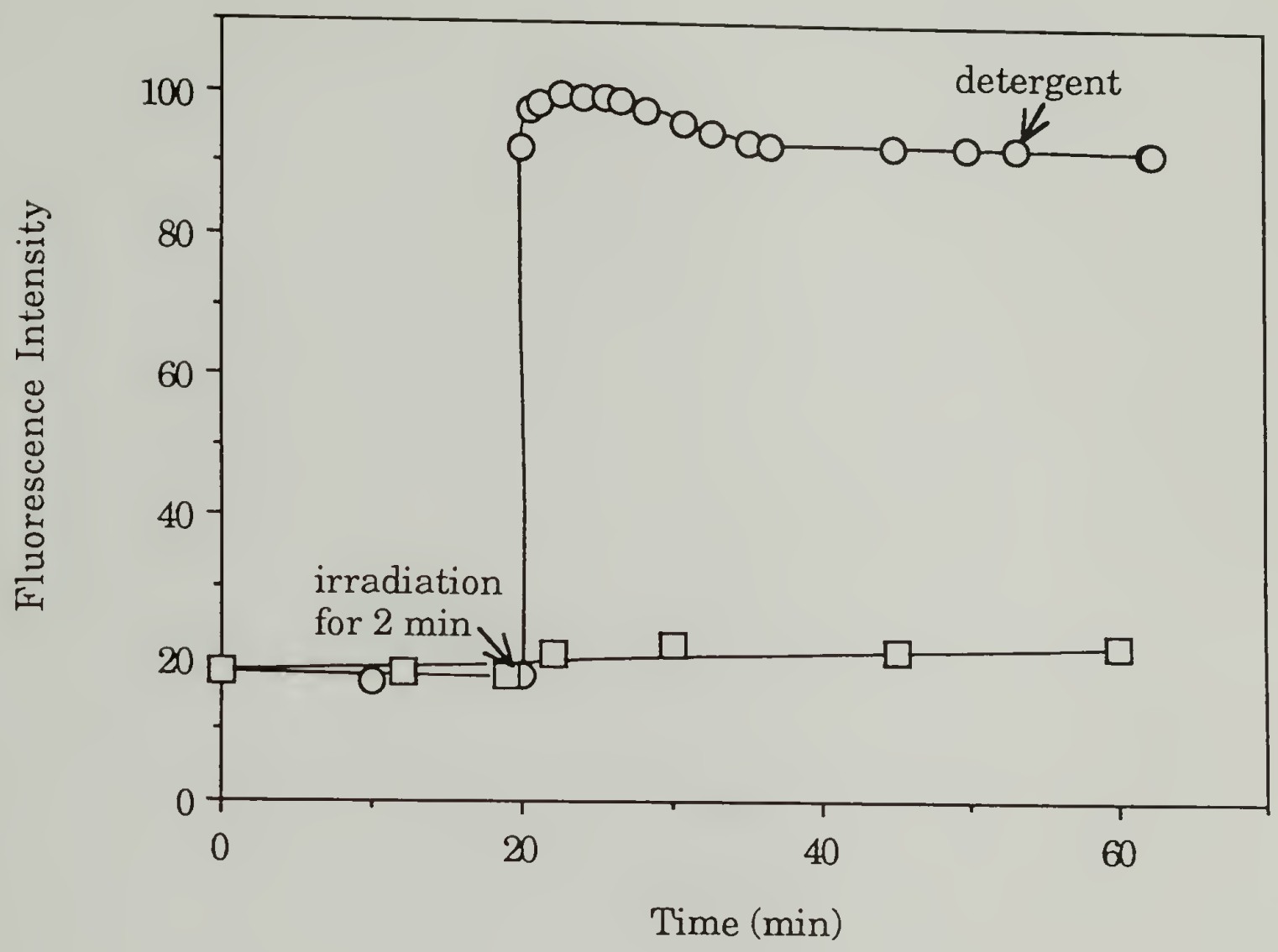


Figure 4.12 Fluorescence intensity of calcein released from sonicated egg yolk phosphatidylcholine (EYPC) (0.038 mg/ml) vesicles by PEAA (0.2 mg/ml) suspended in 1 mM tris buffer (1.6 mM 3,3'-dicarboxydiphenyliodonium bisulfate, 100 mM NaCl) after irradiation for 2 minutes; (○), PEAA; (□), polymer-free control. The initial pH of sample was 7.7 and the final pH after the irradiation was 6.69 at 25 ± 0.2 °C.

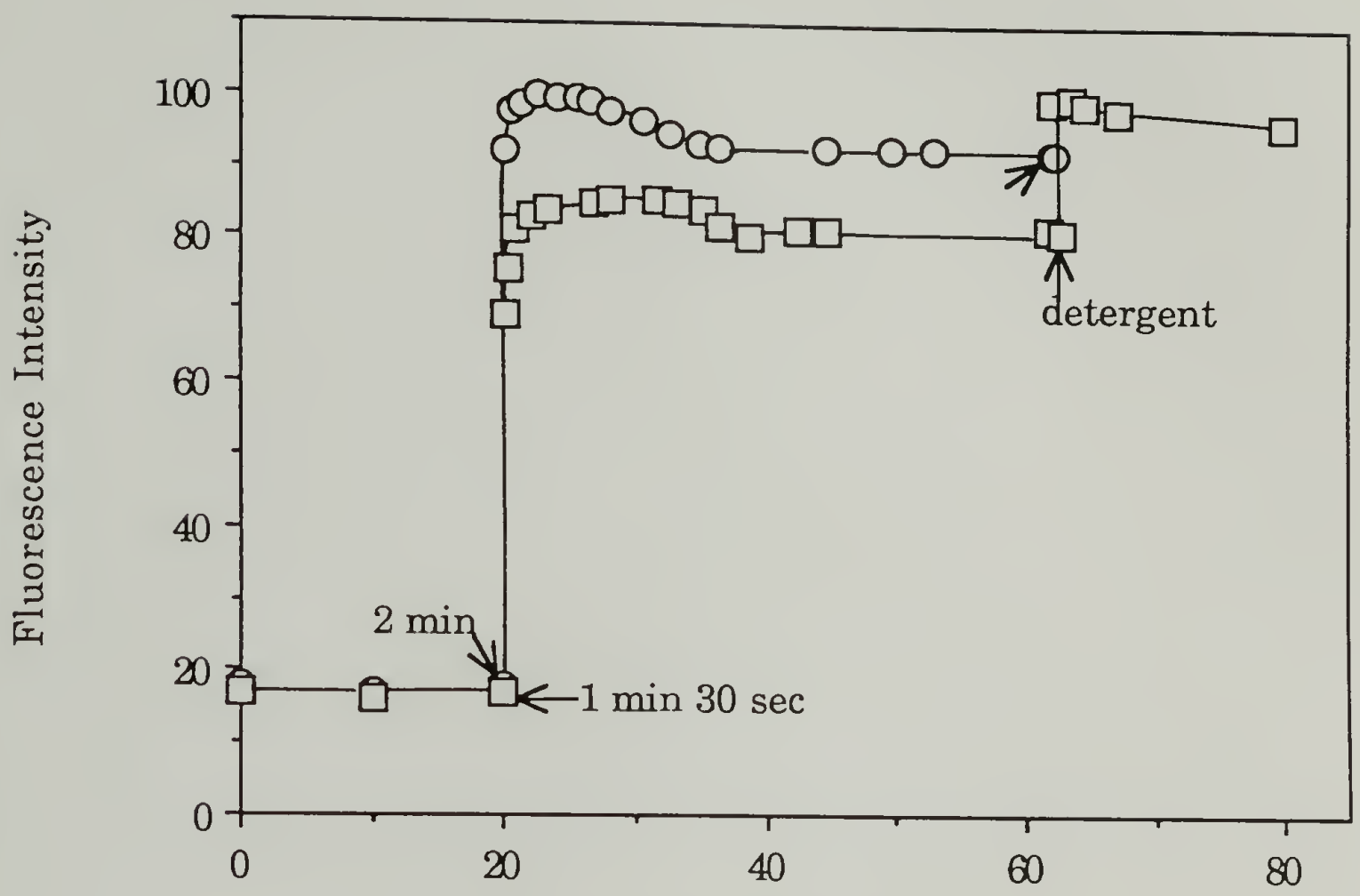


Figure 4.13 Fluorescence intensity of calcein released from sonicated egg yolk phosphatidylcholine (EYPC) (0.038 mg/ml) vesicles by PEAA (0.2 mg/ml) suspended in 1 mM tris buffer (1.6 mM 3,3'-dicarboxydiphenyliodonium bisulfate, 100 mM NaCl) after irradiation for different time periods; (□), 1 minute and 30 seconds; (○), 2 minutes.

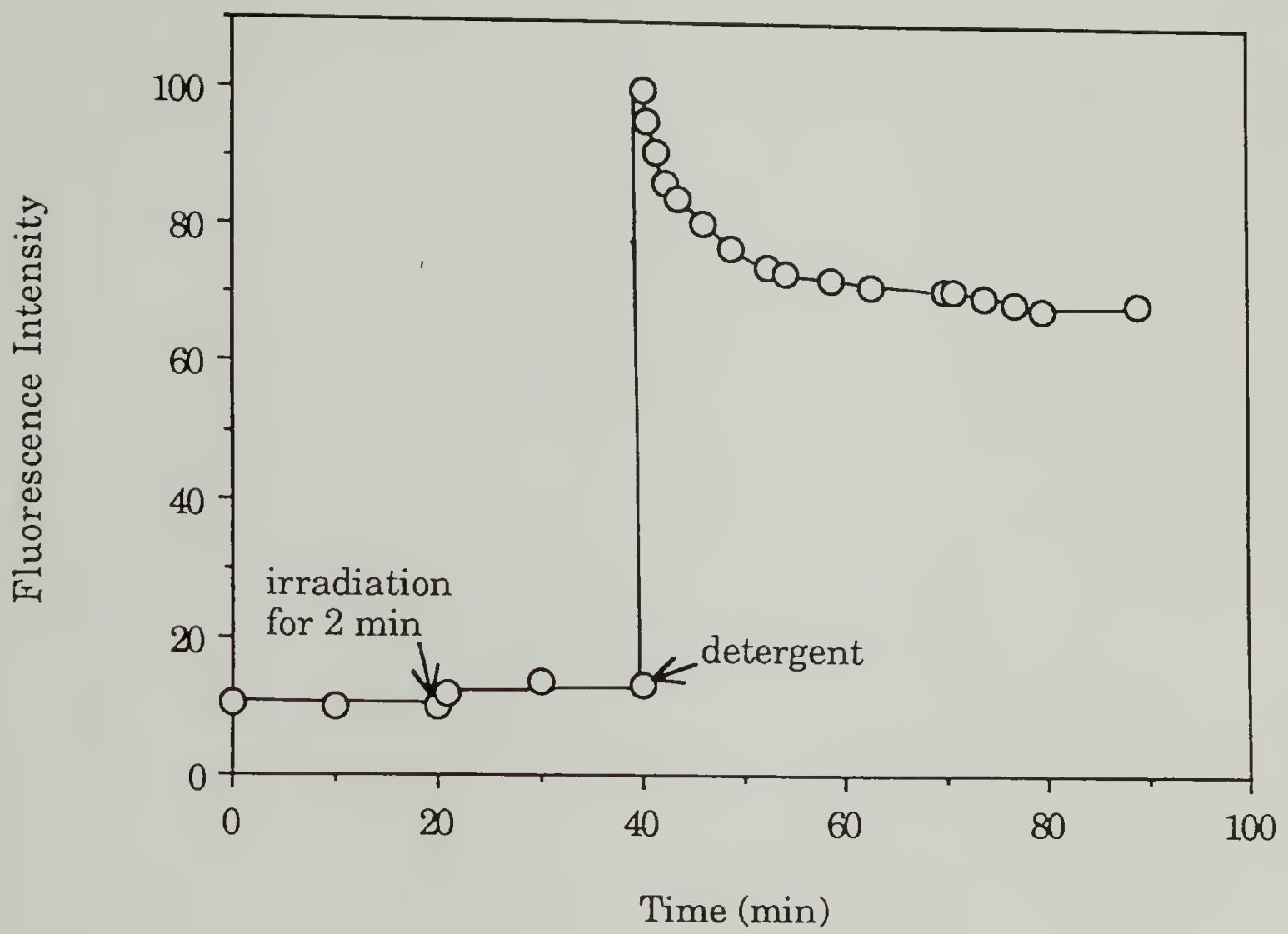


Figure 4.14 Fluorescence intensity of calcein released by Triton X-100 from sonicated egg yolk phosphatidylcholine (EYPC) (0.038 mg/ml) vesicles suspended in 1 mM tris buffer (1.6 mM 3,3'-dicarboxydiphenyliodonium bisulfate, 100 mM NaCl) after irradiation for 2 minutes.

release of dye from EYPC SUV were shown successfully upon irradiation instead of adding acid solution.

A final note on the results of the dye-release concerns certain decreases of the fluorescence intensity after reaching the maximum intensity in the cases of the samples irradiated. As shown in Figure 4.14, addition of detergent results in complete release of dye immediately; the fluorescence intensity reaches a maximum, then decreases over a period of about eight minutes, before levelling off. Addition of the detergent to the same sample without irradiation did not show such a significant drop in fluorescence intensity. Therefore, there seemed to be an effect from the irradiated iodonium salt. In order to investigate the origin of the decrease in fluorescence intensity, a sample of vesicles disrupted by detergent and exhibiting maximum fluorescence intensity was prepared. Control samples of dye-loaded EYPC vesicles and of the iodonium salt alone were irradiated for 2 minutes. The irradiated iodonium salt solution was mixed with an equal volume of disrupted vesicle sample and the irradiated vesicle solution after disruption was combined with an equal volume of fresh buffer solution to give the same concentration of calcein in both preparations. The fluorescence intensities were compared and it was found that a significant decrease of fluorescence intensity was observed in the former preparation compared to the latter. Thus, it is concluded that photolysis of iodonium salt causes drop in fluorescence intensity. Some kind of rearrangment of the disrupted vesicles by Triton X-100 was suggested by Tirrell (32) as a possible origin to cause such a drop of fluorescence intensity. When vesicles containing calcein were disrupted by Triton X-100 a little drop of fluorescence intensity after reaching the maximum value

was observed; however the drop of fluorescence intensity was much less than that observed in the solution of iodonium salt. It is likely that the products of irradiation quench the fluorescence of calcein. The decrease in fluorescence intensity in Figure 4.13, is relatively slow and the fluorescence intensity reached its maximum late compared to the experiment shown in Figure 4.14. In the presence of polymer, during irradiation release of dye takes place with producing the photolyzed products from iodonium salt. As discussed above, increasing of fluorescence intensity and quenching of fluorescence in the solution take place at the same time. Therefore, after a sudden rise in the fluorescence intensity a slow increase is continued until a maximum value after which the release of dye stops, and then fluorescence intensity gradually decreases to a constant value. In the part of the curve showing slow increase of fluorescence intensity, the rate of increase of fluorescence intensity is predominant over that of quenching. On the contrary, in the later stage of gradual drop only quenching of fluorescence is taking place by the products after photolysis of iodonium salt since the release of dye is already complete. This result could be checked from the results of irradiations for different time periods. In Figure 4.13 the fluorescence intensity reaches the maximum value faster with irradiation for longer time than that for shorter time and more drop in fluorescence intensity is also observed in the former case. A final note on this result is the quite long time periods for which the quenching of dye was taking place since in most cases, quenching of fluorescent dye occurs rapidly. Thus, it is suggested that the quenching effect observed here may not be an ordinary one and that

physical phenomena, such as rearrangements of lipid aggregates may be of importance.

E. Conclusions

Photosensitive 3, 3'-dicarboxydiphenyliodonium bisulfate, hexafluorophosphate, and iodide were prepared. Strong acids (H_2SO_4, HPF_6 and HI) were produced upon irradiation at 254 nm and large pH changes in the solution were observed. Structural reorganization of dimyristroylphosphatidylcholine (DMPC) and dilauroylphosphatidylcholine (DLPC) vesicle membranes by poly (2-ethylacrylic acid) in the presence of the iodonium hexafluorophosphate salt was successfully demonstrated upon irradiation . The reorganization of vesicle membrane structures was also detected by monitoring the efflux of entrapped calcein from egg yolk phosphatidylcholine (EYPC) SUV membranes by poly(2-ethylacrylic acid) in the presence of the diaryliodonium salt. There was quantitative release of dye upon irradiation in PEAA solution while diaryliodonium salt itself had no effect on release of dye.

F. References

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CHAPTER V

ENHANCEMENT OF THE CYTOTOXICITY OF IMMUNOTOXINS BY COPOLYMERS OF EAA AND MAA

A. Abstract

Copolymers of 2-ethylacrylic acid and methacrylic acid were employed in specific enhancement of cytotoxicity of immunotoxins. It was found that the copolymer of 49 mole % EAA content (7E copolymer) was not toxic for HeLa cells and potentiates the action of 5E7-gelonin. The potentiating effect gradually increases and then decreases with the increase of the mole fraction of 2-ethylacrylic acid in the polymer. To improve the potentiating effect the 7E copolymer was modified to incorporate 0.9 and 1.6 mole % of 2-pyridyl-2'-aminoethanethiol disulfide in the side chain for attachment to the 5E7-gelonin molecule.

B. Introduction

Goldmacher et al (1, 2) have shown that antibody-complement-mediated cytotoxicity is dramatically enhanced by the ribosome-inactivating proteins gelonin and pokeweed antiviral protein from seeds (PAP-S). They reasoned that it might be possible to prevent the survival of complement-damaged cells by adding to the medium an agent that is not toxic to the

intact cells but that would be highly toxic if allowed to gain access to the cell cytoplasm. The ribosome-inactivating proteins gelonin (3) and PAP-S (4) appeared to be agents that fit this description. These molecules inhibit protein synthesis in a cell-free system at a concentration range of 10 to 100 pM, but are essentially nontoxic to intact cells even at 0.1 to 1 μM (3, 4).

It has previously been established that, in general, conjugates of single-chain ribosome-inactivating proteins (e.g., gelonin) with antibodies targeted towards cell surface antigens are not efficient in penetrating cellular membranes. As a result, such conjugates are not highly cytotoxic for target cells.

Tirrell and coworkers have developed a series of copolymers of EAA and MAA. These copolymers destroy phospholipid vesicle membranes at acidic pH, but not at neutral pH (see Chapter III). Endocytosis is an obligatory step in the cell intoxication by gelonin conjugates, and endosomes are quickly acidified upon their formation during the cojugation process (5). Therefore, one might hope that the EAA-MAA copolymers would destroy endosomal membranes, but not plasma membranes, and would not be toxic. If endosomes contain a gelonin conjugate, then, upon the destruction of the endosome, the conjugate will be released into cytoplasm, where it will be able to reach its target, the ribosome. In other words, the behavior of copolymers destroying lipid membranes at acid pH might be used for specific enhancement of cytotoxicity of immunotoxins.

In a collaboration with Immunogen Inc.(Cambridge, MA), the copolymers of EAA and MAA, and PEAA were applied for the enhancement of the cytotoxicity of immunotoxins and 7E copolymer was modified to improve the potentiating effect.

C. Experimental

1. Materials

All reagents and their sources are listed below. The reagents were used as received unless otherwise stated.

Acetic acid, A.C.S. reagent (A)

Acetic acid, glacial (A)

2-Aminoethanethiol hydrochloride (cysteamine) (A)

Benzene, HPLC grade (A)

Calf serum supplemented with iron (F)

Cellulose dialysis tubing, Spectra / Por 6, molecular weight 1000 cutoff, rinsed with methanol prior to use. (F)

Chloroform, HPLC grade (F)

Deutrium oxide (D_2O) (A)

1,3-Dicyclohexylcarbodiimide (DCC) (A)

N,N-Dimethylformamide (DMF) (A)

2,2'-Dipyridyl disulfide (Aldrithiol-2) (A)

Dithiothreitol (DTT; threo-1,4-dimercapto-2,3-butanediol) (A)

Ethanol, absolute (P)

Ethyl acetate (A)

Ethyl ether (A)

Gelonin cojugate

L-Glutamine (G)

HeLa cell (Fl)

Namalwa cell (Fl)

RPMI medium (G)

Silicagel (230-300 mesh size) (F)

Sources

(A) Aldrich Chemical Co. (Milwaukee, WI)

(F) Fisher Scientific (Boston, MA)

(Fl) Flow Laboratories Inc.(Mclean, VA)

(G) GIBCO Laboratories (Grand Island, NY)

(P) Pharmco Products (Dayton, NJ)

2. Cytotoxicity measurement (5)

Cytoxicity measurements were performed at Immunogen, Inc., Cambridge, MA. Procedures are provided in a private communication from V. Goldmacher (5).

3. Modification of 7E copolymer

a. Synthesis of 2-aminoethyl-2'-pyridyl disulfide hydrochloride.

2,2'-Dithiopyridine (4.48 g, 20 mmol) was dissolved in 14 mL of absolute ethanol and 960 mL of glacial acetic acid was added. 2-Aminoethanethiol hydrochloride solution was prepared by dissolving 1.136 g (1 mmol) of 2-aminoethanethiol hydrochloride in 8 mL of ethanol. This solution was added to the 2,2'-dithiopyridine solution by a dropwise addition with vigorous sttiring. The initial reaction mixture was green-to-yellow and

clear. The reaction flask was wrapped with aluminum foil to prevent the light from affecting the reaction. The reaction was stopped after 48 hours and precipitated material was separated from the solution part by centrifugation of the reaction mixture for 20 minutes in an IEC Model CL Clinical Centrifuge with a 4 x 50 ml swinging bucket rotor (3350 rpm max) at setting 5 (~ 2500 rpm). Exhaustive evaporation of the solvent left a viscous yellow liquid. The viscous liquid diluted with solvent A as below was applied to a column (Silicagel 230 to 300 mesh size, 2 x 20 cm) for separation of product using eluents as follows; about 440 ml of solvent A (chloroform : benzene : acetic acid = 3/1/0.04) was eluted until all visible yellow color disappeared to separate 2-thiopyridine mainly and a little 2,2'-dithiodipyridine, 300 ml of solvent B (chloroform : benzene : acetic acid = 3/1/0.4) to separate mostly 2,2'-dithiodipyridine and a little of 2-thiopyridine, and finally 340 ml of solvent C (chloroform : acetic acid = 1/1) to separate most of product. After reducing the volume of each fraction, thin layer chromatography (TLC) R_f values were measured (Fraction A : R_f = 0.2 for 2-thiopyridine and R_f = 0.6 for 2, 2'-dithiodipyridine, Fraction B : R_f = 0.52 for 2-thiopyridine and R_f = 0.68 for 2, 2'-dithiodipyridine, and R_f = 0.1 for product, and the R_f values measured herein were similar to those reported (6). The developing solvents for TLC were the same as those employed in column separation). After evaporation of solvent of fraction C, pale yellow crystal product was obtained. By recrystallization in ethanol, white powdered crystal of 2-aminoethyl-2'-pyridyl disulfide hydrochloride was collected and dried under vacuum to give about 60 % yield.

b. Coupling of 2-aminoethyl-2'-pyridyl disulfide hydrochloride with 7E copolymer. 7E copolymer was dissolved in 1.5 ml of anhydrous DMF to give 55 mM polymer solution in a small vial. DMF solution of DCC was added, and disulfide compound and triethylamine in anhydrous DMF were then combined to the reaction mixture (amount of each chemical used in the reaction is summarized in Results and Discussion part). The reaction was carried out in a vial wrapped with aluminum foil at room temperature. After 36 hours the reaction mixture was poured into a large volume of ethyl acetate to precipitate the polymer sample. After filtering the polymer, precipitated polymer was again dissolved in methanol. The precipitation was repeated in ethyl acetate and the polymer sample was filtered. After dissolving polymer sample in methanol the polymer solution was filtered, placed in cellulose dialysis tubing (Spectra/Por 6, 1000 MW cutoff), and dialyzed against methanol for 48 hours. The external methanol was replaced with fresh solvent every 12 hours. The polymer was finally precipitated in ethyl ether by a dropwise addition, filtered, and then dried under vacuum.

c. Measurements. ^1H NMR spectra of 2-aminoethyl-2'-pyridyl disulfide were recorded on a Varian XL-300 NMR spectrometer with D_2O as a solvent. UV spectra of the 2,2'-dipyridyl disulfide aqueous solution (1.05×10^{-4} M) in the presence of dithiothreitol were recorded on a Beckmann DU-7 UV/VIS spectrophotometer. The extinction coefficient (ε) of 2-thiopyridone at $\lambda = 343$ nm was determined to be $\varepsilon = 8.16 \times 10^3\ M^{-1}\ cm^{-1}$ (error $\pm$ 3 %) (ε has been reported as 8.08×10^3) (1). Purity of 2-aminoethyl-2'-pyridyl disulfide was checked by determining the extinction coefficient at $\lambda = 343$ nm using dithiothreitol. The extinction coefficient of 2-thiopyridone from

the 2-aminoethyl-2'pyridyl disulfide (1.03×10^{-4} M) with dithiothreitol was the same as that from 2,2'-dipyridyl disulfide. The mole fraction of disulfide linkage (substituted fraction in monomeric units) in modified polymer was calculated by measuring the absorbance at 343 nm after adding dithiothreitol using the extinction coefficient determined here. The extinction coefficients of 2-thiopyridone from 2-aminoethyl-2'-pyridyldisulfide (1.03×10^{-4} M) in aqueous solutions of pH 3.7 and 7.2 were examined, to check any possible effect of solution pH. An aqueous polymer solution (pH 7) was prepared and the content of the disulfide compound in the modified polymer was determined by the same method, using the extinction coefficient of 2-thiopyridine determined here.

D. Results and Discussion

1. Studies on Namalwa cells (5)

It was found that in preliminary tests that 7E copolymer at a concentration of 20 mg/ml is moderately toxic for Namalwa cells (surviving fraction was between 0.3 and 0.5 in different experiments). The cytotoxicity of 5E9 - gelonin for Namalwa cells with or without 7E copolymer was measured and the data are summarized in Figure 5.1. It was found that there is only additive effect of the polymer on the cytotoxicity of 5E9 - golonin and no potentiating effect could be detected.

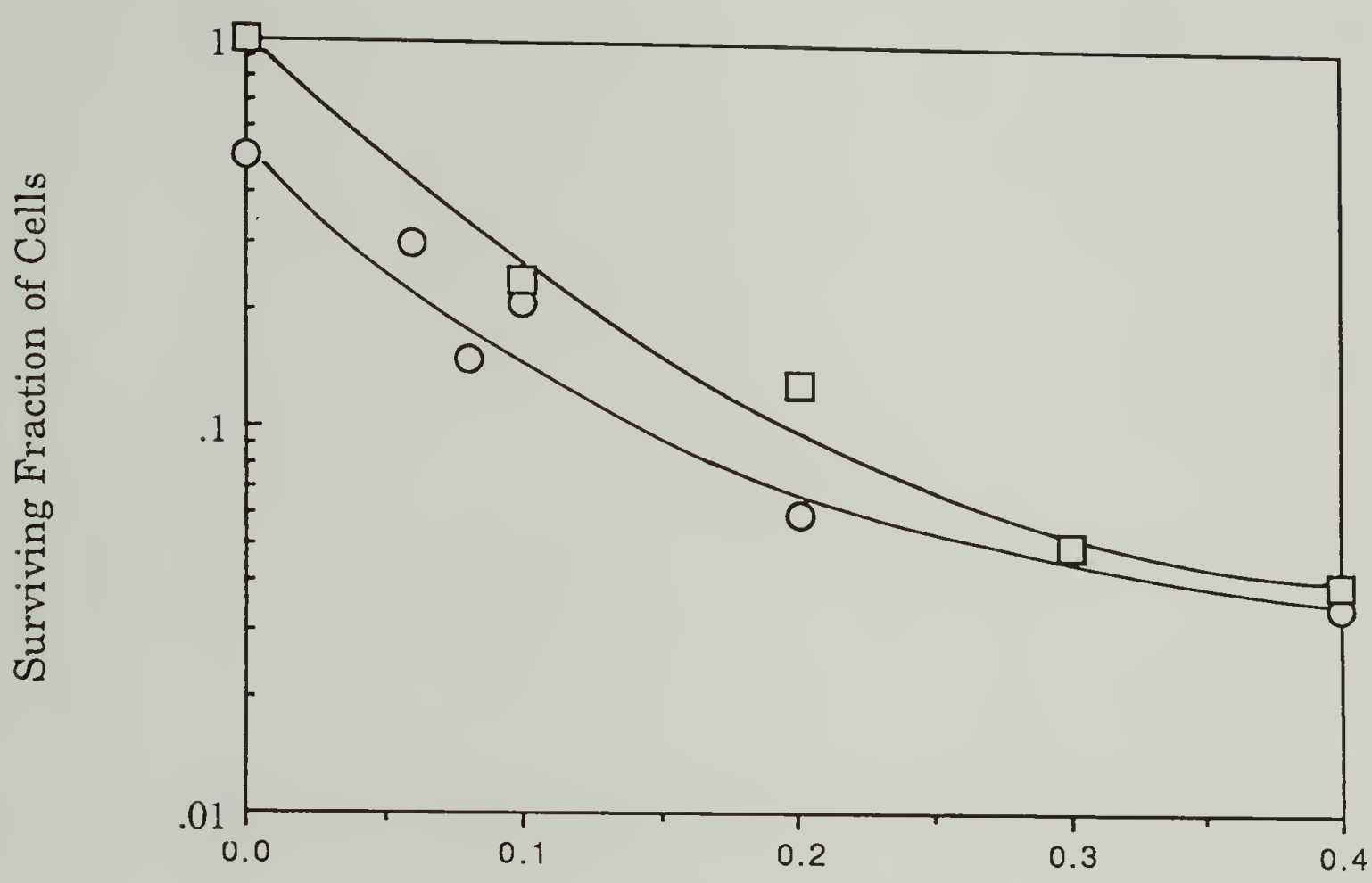


Figure 5.1. Cytotoxicity toward Namalwa cells of 5E9-gelonin with (○) or without (□) 7E copolymer (20 mg/ml).

2. Studies on HeLa cells (5)

In contrast to the Namalwa cells, 7E is not toxic for HeLa cells and does potentiate the action of 5E9 - gelonin as shown in Figure 5.2. Similar experiments were done with the other copolymers and the data are summarized in Table 5.1. IC_{50} (the concentration of 5E9 - gelonin that kills 50% of the cell population) was used as an index of cytotoxicity. Interestingly, the potentiating effect gradually increases and then decreases with the increase of the content of EAA in polymer. 7E copolymer appears to potentiate the cytotoxicity of 5E9 - gelonin most effectively.

3. Modification of 7E copolymer

Based on the results described, a more pronounced potentiating effect was anticipated by preparaing the properly modified polymer in order to attach the polymer covalently to the 5E9-gelonin molecule. Then, it would provide much higher local concentration of polymer in the vicinity of the membrane-bound immunotoxin while the non-specific toxicity of the conjugated polymer would be reduced since its overall concentration would be low.

A synthetic scheme for modification of polymer is drawn in scheme 5.1. The coupling agent having disulfide linkage has been known to be a good candidate for coupling with other sites, such as cysteine in proteins. As a precursor for the coupling agent, 2-aminoethyl-2'-pyridyldisulfide (as its hydrochloride salt) was synthesized and characterized by ^1H NMR spectoscopy. In Figure 5.3, the two methylene protons adjacent to the disulfide are shown at about 2.15 ppm and the neighboring methylene

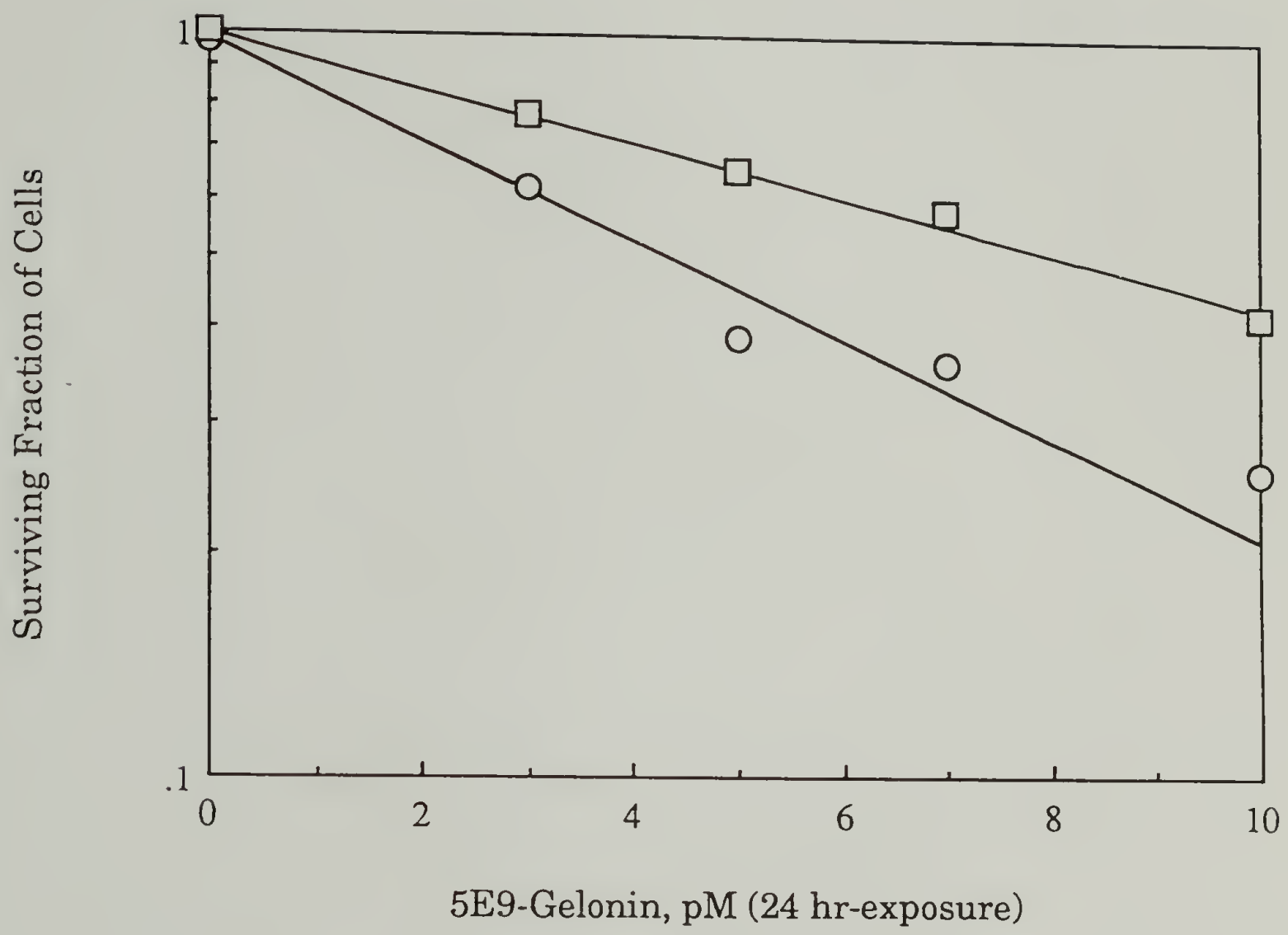
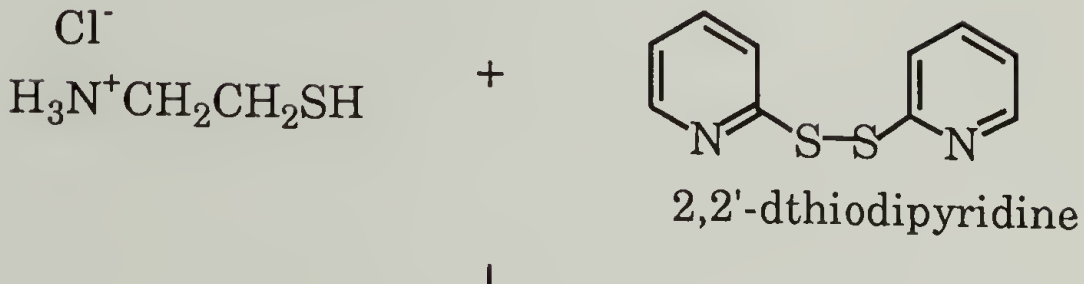


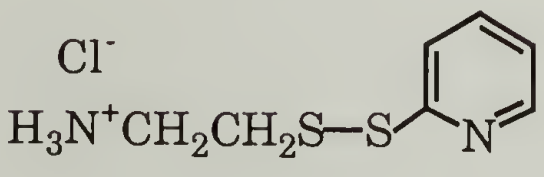
Figure 5.2. Cytotoxicity toward Hela cells of 5E9-gelonin with (○) or without (□) 7E copolymer (20 mg/ml).

Table 5.1. Cytotoxicity of 5E9-gelonin for HeLa cells in the presence of polymers.

Polymer in Medium (0.02 mg/ml)	MAA mole % in Copolymer	IC_{50} (pM)
none		16
PMAA	100	36
2E	16	6.3
3E	22	7.3
5E	65	5.7
7E	49	4.4
8E	40	16
PEAA	0	22

Scheme 5.1 Modification of 7E copolymer.





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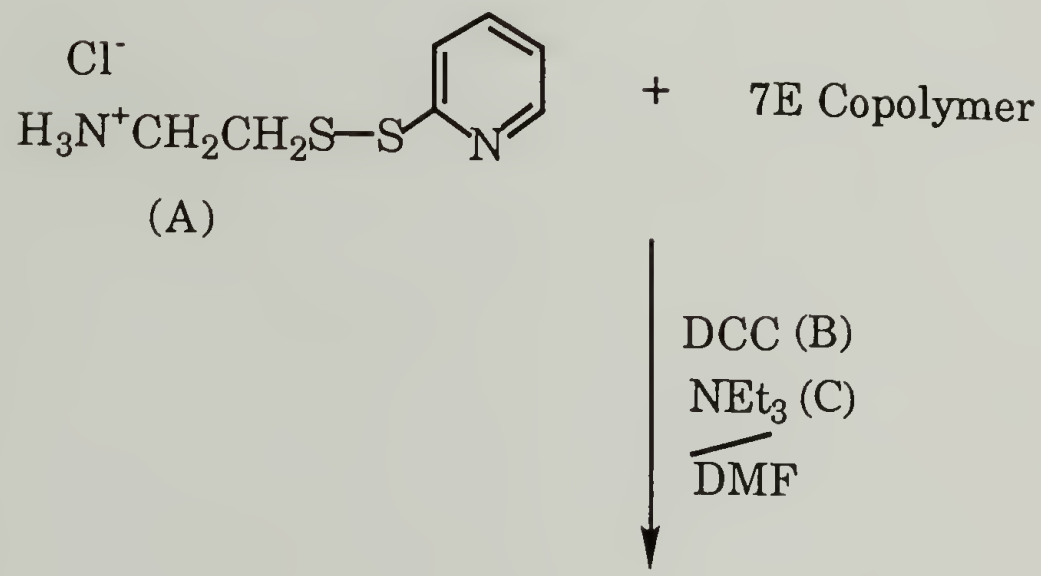
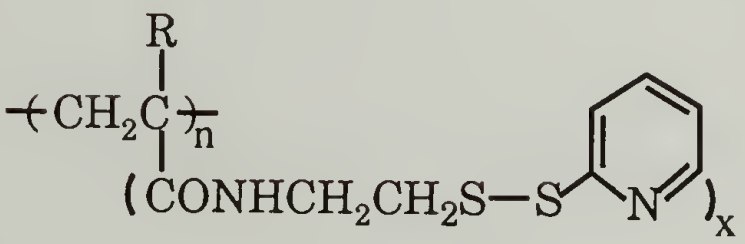
Scheme 5.1 continued





R = CH_3(51 %) and C_2H_5(49 %)

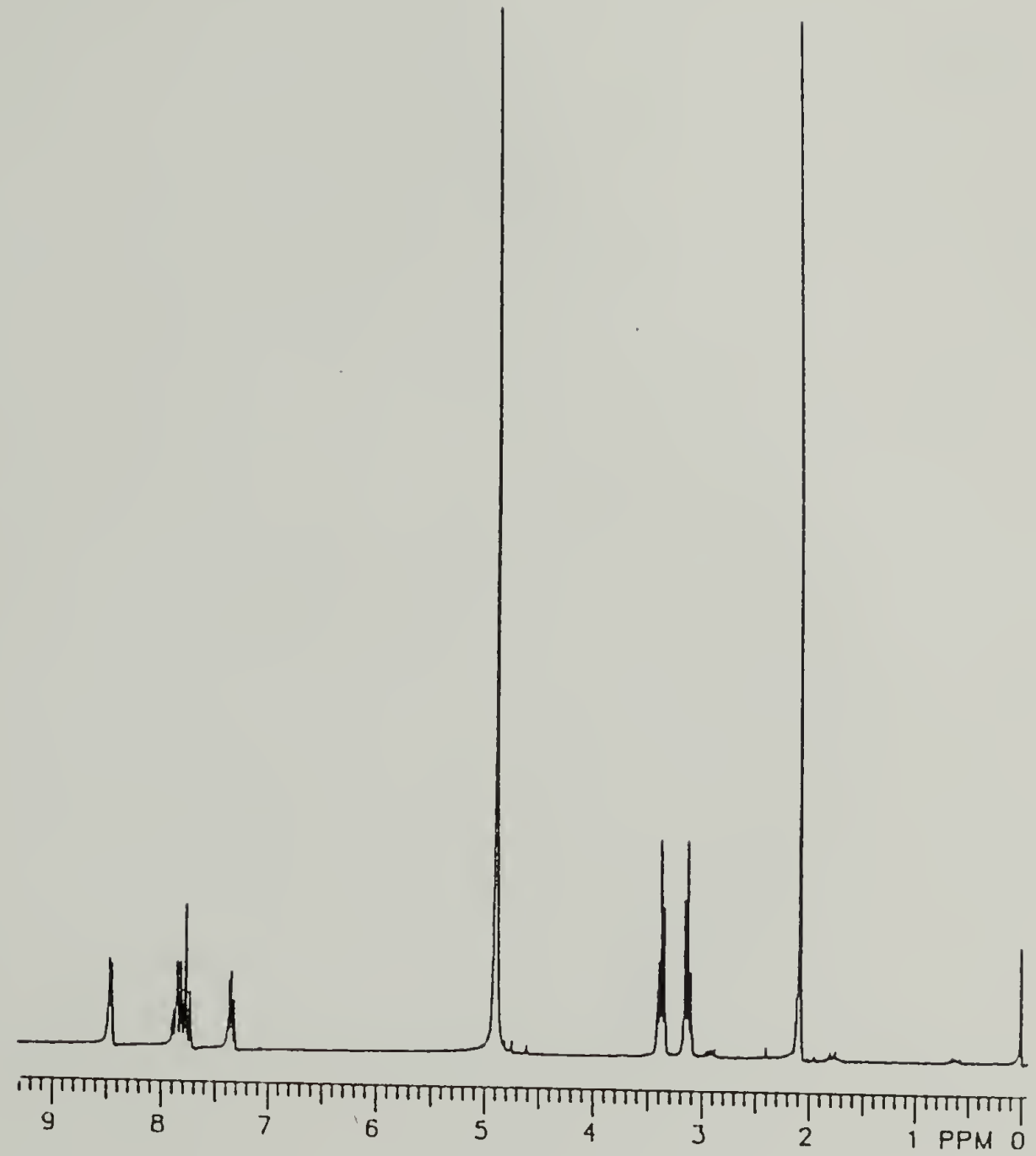


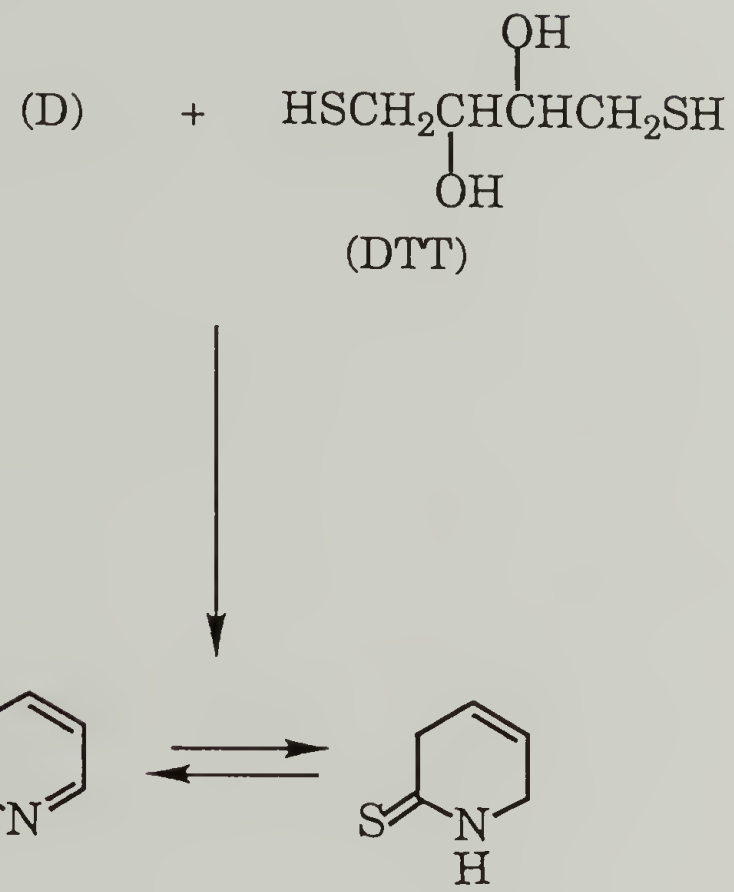
Figure 5.3 ^{1}H NMR spectrum of 2-aminoethyl-2'-pyridyl disulfide in D_2O at 18 °C.

protons at about 3.3 ppm. The protons in the aromatic ring are shown at 7.85, 7.75, 7.3, and 8.5 ppm, proceeding from the proton next to the nitrogen to rest of protons in a counter-clockwise direction. The ^1H NMR spectrum shows the compound to be very pure.

The procedure used for determination of SH groups in the modified polymers is drawn in scheme 5.2 (6,7). The procedure utilizes the reaction of 2,2'-dipyridyl disulfide with thiols, which gives the corresponding 2-thiopyridone which is almost exclusively in the tautomeric form with the mobile hydrogen attached to the nitrogen (7). This causes the ultraviolet absorption spectrum of thiopyridone to be quite different from those the corresponding disulfides. The method permits determination of thiol quantitatively, and the formation of 2-thiopyridone and the disappearance of the disulfide can be followed.

UV spectra of the 2-2'-dipyridyldisulfide aqueous solution (1.05×10^{-4} M) and those of the sample after adding dithiothreitol after different time period are shown in Figure 5.4. As reported in the literature (7, 8) λ_m of 2-thiopyridone was found at 343 nm and increased with time. Using 2,2'-dipyridyl disulfide, the extinction coefficient was determined to be $\varepsilon = 8.16 \times 10^3\ M^{-1}\ cm^{-1}$ (error $\pm$ 3 %) which corresponds to the reported value, 8.08×10^3 (2) (see Scheme 5.2). The extinction coefficient measured from 2-aminoethyl-2'-pyridyl disulfide was the same as that from 2,2'-dipyridyl disulfide. This result again confirmed the purity of this compound. At different pH 3.7 and 7.2, the extinction coefficients were calculated to be almost identical.

Scheme 5.2 Determination of concentration of 2-thiopyridone.



The extinction coefficient of 2-thiopyridone was determined to be 8.16 x 10^3 M^{-1} cm^{-1} (error ± 3 %).

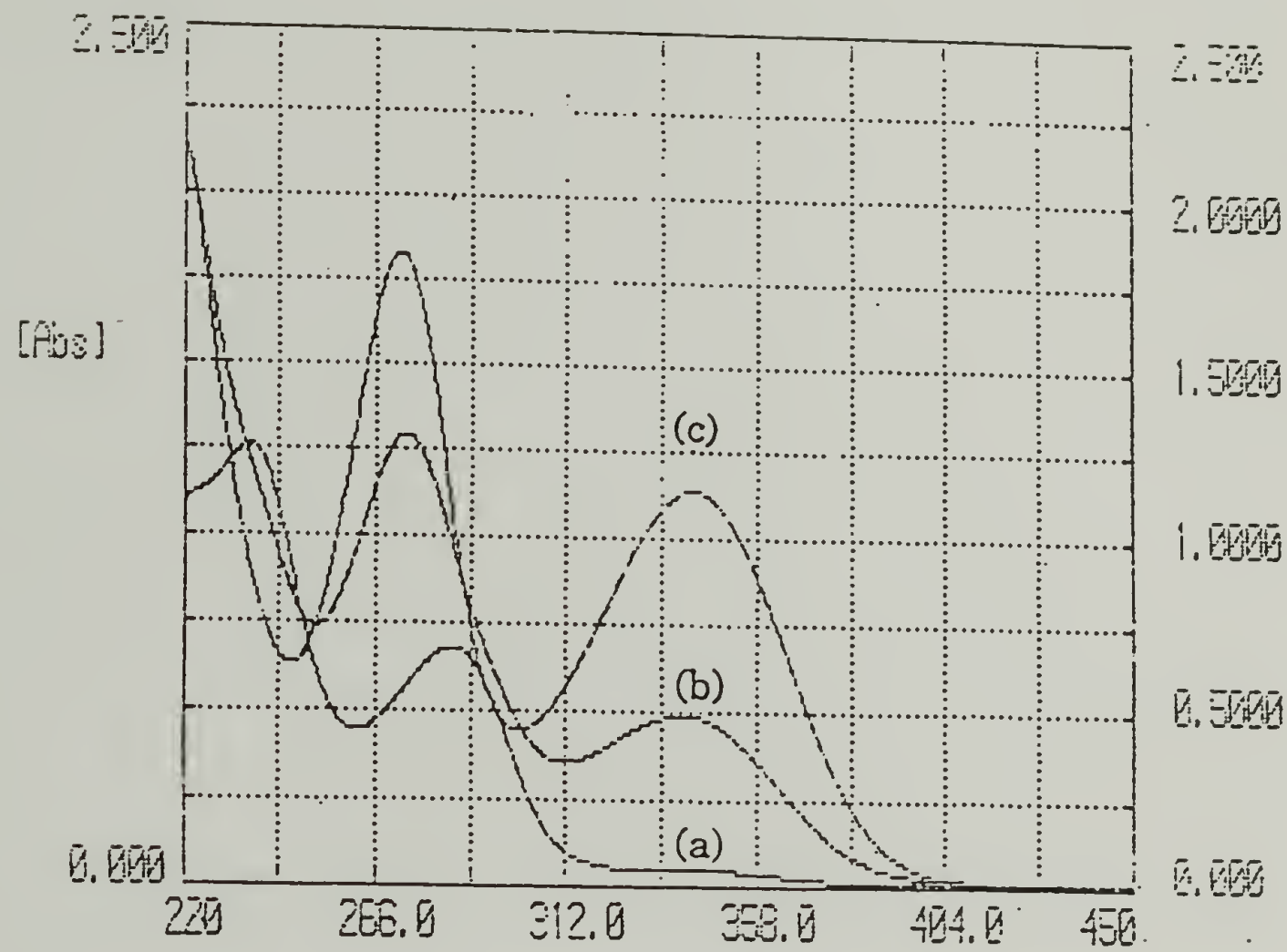


Figure 5.4 Ultraviolet spectra of (a) 2,2'-dipyridyl disulfide ($1.03/2 \times 10^{-4}$ M), and (b) and (c) at different time (after 15 sec and 60 sec, respectively) after addition of dithiothrietol in aqueous solution.

Table 5. 2. Mole % of modified side chain (x) based on monomer repeat unit in 7E copolymer.

Mole % on the basis of monomer repeat unit		
APD[a]	DCC	(X)
9	3.5	1.6
9	2	0.9

[a] 2-aminoethyl-2'-pyridyl disulfide hydrochloride

The modified polymer as drawn in synthetic scheme 5.1 was obtained with varying amounts of disulfide groups depending on the ratios of reagents used. The results are summarized in Table 5.2.

E. Conclusions

The specific enhancement of cytotoxicity of immunotoxins was observed by using the copolymers of 2-ethylacrylic acid and methacrylic acid. The potentiating effect gradually increased and then decreased with the increase of the mole fraction of 2-ethylacrylic acid in the polymer. 7E copolymer was not toxic for HeLa cells and appeared to potentiate the cytotoxicity of 5E7-gelonin most strongly. The 7E copolymer was modified to have 0.9 and 1.6 mole % of disulfide group in the side chain for attachment to the 5E7-gelonin molecule in order to enhance more the potentiating effect. Covalent attachment to the immunotoxin should provide much higher local concentration of polymer in the vicinity of the membrane-bound immunotoxin and should lower the non-specific toxicity of the conjugated polymer.

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CHAPTER VI

CONCLUSIONS

A. Summary

We have prepared copolymers of 2-ethylacrylic acid and methacrylic acid in bulk and in DMF. The reactivity ratios of monomers were estimated by fitting copolymerization data to the composition equation derived for the terminal copolymerization scheme. With increasing amounts of DMF in copolymerization mixtures (from bulk to 75 % DMF solution), copolymers richer in MAA were produced from the same monomer feeds. Partitioning of monomers between the domains of copolymer radicals and the solvent was suggested the origin of this result. ^{13}C NMR spectra provided convincing evidence for the formation of statistical copolymers. Increases in the EAA content of the reaction mixture were accompanied by reduction in the apparent molecular weights of the copolymers.

We have found that the pH-dependent structural reorganization of DPPC vesicle membranes suspended in aqueous buffer solutions of EAA-MAA copolymers is sensitive to the composition of the copolymer. The shift in critical pH was significant until the EAA content in the copolymer was decreased to 49 mole%; compared to the critical pH of 6.55 for PEAA, that for copolymer containing 49 mole % EAA was shifted to pH 5.65. However, with the copolymers of EAA content equal to or less than 40 mole% , there was no clarification of DPPC vesicle suspensions and complicated

aggregation was observed. The shifts in the critical pH are attributed to the different hydrophobic interactions of polymers with the membrane surface. It is concluded that the ionization at which the conformational transition or the disruption process takes place is shifted to higher values for PEAA compared to the copolymers, as a result of stronger hydrophobic interactions in the former compound.

Even those copolymers which did not cause disruption of the DPPC vesicles induced the release of dye from EYPC SUV membranes. The different hydrophobic interactions of polymers were again demonstrated by showing complete or partial release of dye depending on the hydrophobicities of polymers. Modified PMAA having 14 % of n-hexyl side chains exhibited the partial release in a relatively wide range of pH. This seems to be due to the intrinsic hydrophobic nature of the n-hexyl side chains regardless of pH; therefore this polymer is not as pH-sensitive as the other polymers.

Photosensitive 3, 3'-dicarboxydiphenyliodonium bisulfate, hexafluorophosphate, and iodide have been prepared. Strong acids (H_2SO_4, HPF_6 and HI) were produced upon irradiation at 254 nm and large pH changes in solution were observed. Structural reorganization of dimyristroylphosphatidylcholine (DMPC) and dilauroylphosphatidylcholine (DLPC) vesicle membranes by poly (2-ethylacrylic acid) in the presence of the iodonium salts was successfully demonstrated upon irradiation. The reorganization of vesicle membrane structures was also detected by monitoring the efflux of entrapped calcein from EYPC SUV membranes by poly(2-ethylacrylic acid) in the presence of diaryliodonium salts. There was quantitative release of dye upon

irradiation while the diaryliodonium salt itself had no effect on release of dye.

The specific enhancement of cytotoxicity of immunotoxins was observed by using the copolymers of 2-ethylacrylic acid and methacrylic acid. The potentiating effect gradually increased and then decreased with increasing mole fraction of 2-ethylacrylic acid in the polymer. The copolymer containing 49 mole% EAA was not toxic for HeLa cells and appeared to potentiate the cytotoxicity of 5E7-gelonin the best. This copolymer was modified to have 0.9 and 1.6 mole % of disulfide group in the side chain for attachment to the 5E7-gelonin molecule in order to enhance further the potentiating effect.

B. Future Work

Copolymers of EAA and MAA prepared in bulk were used in the study of phospholipid vesicle membranes as described in this work. In addition, copolymers from AA and MAA were prepared by Chapiro et al.. Therefore it would be of interest to synthesize new poly(carboxylic acid)s from AA and EAA. From the copolymerization of AA and EAA, it is also anticipated to obtain the statistical copolymers which have useful conformational properties since the monomer reactivity is highest for MAA among those monomers.

Although the detailed information on the monomer sequence distribution in copolymers of EAA and MAA was left unanswered, it might be investigated by modification of copolymers. Seki and Tirrell showed the

tacticities of PEAA by methylation of PEAA to methyl ester of PEAA (1). Therefore, more accurate sequence distribution might be determined by obtaining better resolved NMR spectra.

It has been discussed in Chapter III that in the structural reorganization of DPPC vesicle membranes, we saw the effects of the different hydrophobicities of the polymers by showing the shifts in the critical pH. It would be also of interest to have the same phenomena probed in different ways. The best way is to study the kinetics of optical density changes of DPPC vesicle samples at various pH. Actually, the 49 mole% EAA copolymer needed longer times to disrupt DPPC vesicles than did PEAA. Since we observed differences in the slopes in the plot of pH versus optical density, it may also be possible to obtain more data points near to joint region between the plateau and the cureve (or transition) region in order to show more clear differences in the slopes.

From the results of the structural reorganization of DPPC vesicle membranes and of the release of dye by copolymers, it was concluded that the copolymers with less than 49 mole% of EAA were not capable of disrupting DPPC vesicles, but exhibited some hydrophobicities showing the release of dye from EYPC SUV and forming the aggregates at acidic pH. Therefore it would be of interest to employ other phosphatidycholines, such as dimyristroyl or dilauroyl phosphatidylcholine (DMPC or DLPC) which are less stable than DPPC in the interaction with such copolymers.

The copolymer having 40 mole% EAA exhibited complicated behavior in the interaction with DPPC vesicle membranes ; the formation of aggregates in the lipid/polymer mixture at pH 5.2 and the clarification upon increasing pH to 6.2. It was suggested that there might be two

competitive processes at low pH (pH 5.2), i.e., a very slow rate of disruption of vesicles and formation of aggregates. In order to investigate this it would be possible to measure the changes of optical densities of a series of samples after long time period (it may take a month). With those samples after a certain time period, the clarification process could be checked by increasing pH. The clarification process was observed to be dependent upon the time period of heat treatment ; the aggregates were formed after about 20 minutes and if the sample was allowed to stand at low pH for longer time, faster clarification (followed by lower optical density) was observed.

There have been many investigations on the intermolecular hydrophobic interaction of acid polymers that were partially substituted with long alkyl chains or some hydrophobic moieties, such as aromatic compounds (or copolymerized with monomers having long alkyl chains or hydrophobic moieties). Most of these polymers have much longer alkyl chains than ethyl and it has been reported that with a proper concentration of such alkyl chains in the polymer the number of carbon in the alkyl side chain should be more than four. The copolymers of maleic acid with alkyl vinyl ethers with different alkyl groups demonstrated the intermolecular hydrophobic interaction in a certain range of degree of ionization when the alkyl groups consist of more than four carbons.

As mentioned previously, the viscosity as a function of pH has been investigated (1,2,3) for polyelectrolytes. Intramolecular hydrophobic interaction was observed by showing very low viscosity because of the high degree of compactness of polymer chains at low pH (low degree of ionization) compared to that of common polyacids such as poly(acrylic

acid). The increase of viscosity is then observed when the polymer conformational transition takes place, and finally the highest viscosity as a plateau after the degree of ionization is above a certain value.

When the 34 mole% EAA copolymer was dissolved (3~5 mg/ml) in saline buffer solution or in distilled water, extremely high viscosity was observed at around pH 5. The polymer solution in the vial did not flow when the vial was turned upside down and even shaken. However, upon increasing pH above 6.5 the viscosity dropped to a value similar to those of the other polymer solutions (e.g., PEAA or the 49 mole% copolymer) with the same polymer concentration. In the case of PEAA, PMAA, and copolymers such a behavior in viscosity was not observed before. We suggest this result to be originated from the different hydrophobicities of the polymers. The viscosities of PEAA and PMAA solutions at high concentration of polymer (3~5 mg/ml, about 0.03~0.05 N) have not been reported and those results described on behavior of viscosity were obtained at low concentration (0.01~0.0025 N). However, even at high concentrations, these polymers did not exhibit the behavior in viscosity as the 34 mole% EAA copolymer. For PEAA the polymer chain is hydrophobic enough to collapse by intramolecular hydrophobic interaction but for the 34 mole% copolymer the hydrophobicity is proper to induce the intermolecular interaction before the intramolecular interaction takes place at low pH when the polymer concentration is high enough. In order to investigate the result in detail it is necessary to measure the viscosities of polymer solutions at different concentrations and also at different pH. We can in this way obtain the critical pH beginning to show high viscosity and the critical concentration of polymer above which the intermolecular

interaction takes place while the intramolecular hydrophobic interaction was already observed at low concentration of polymer.

The release of dye from the EYPC membranes has been in general detected by using a fluorescence spectrophotometer; however, the preparation of "giant" vesicles with oligo-lamellar or unilamellar structure has also been reported (5). In addition, we synthesized in this work iodonium salts that can be applied in the interaction of PEAA with vesicle membranes. Therefore, it would be nice to monitor the release of dye from vesicles with a fluorescence microscope by combining those techniques. Actually, we could prepare very big vesicles of EYPC (over 20 μm in diameter) entrapping calcein inside and easily separate them from the external medium (see APPENDIX). We tried to monitor the release of calcein in the presence of PEAA and iodonium salt upon irradiation, however quenching of the fluorescence intensity of calcein after irradiation made the result complicated. In order to get the maximum fluorescence intensity in vesicles 3 to 5 mM calcein was applied but the fluorescence intensity from such a low concentration of calcein bleached by irradiation (in CHAPTER IV, 250 mM calcein was used, thus the high intensity of fluorescence was still observed after quenching). If a proper dye is chosen which is not affected by irradiation in the presence of iodonium salt and well soluble in aqueous solution, then it might be possible to follow the release process. The reorganization process (change in vesicle shapes) of DLPC or DMPC vesicles can also be monitored using iodonium salts under the phase-contrast microscope.

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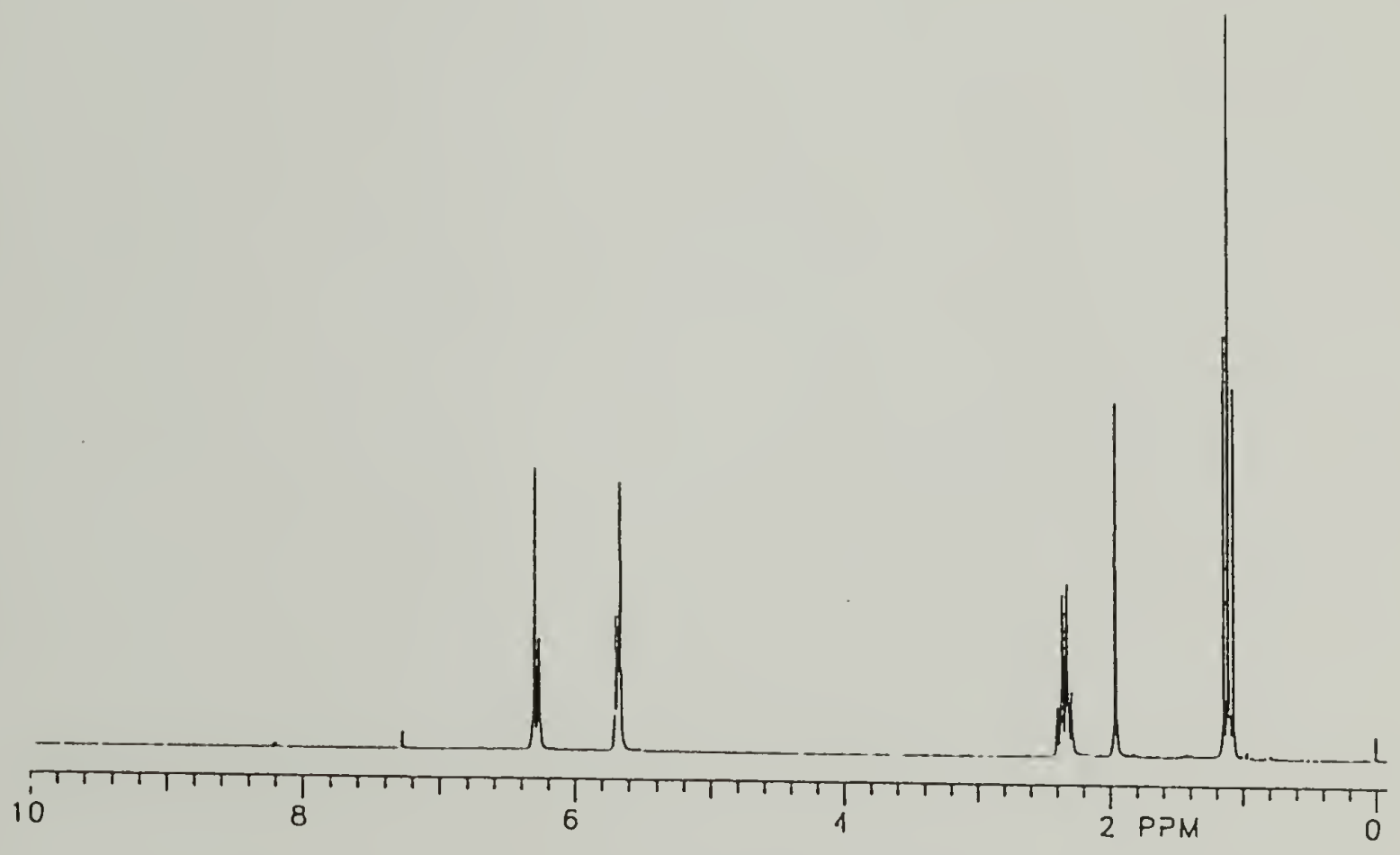
APPENDIX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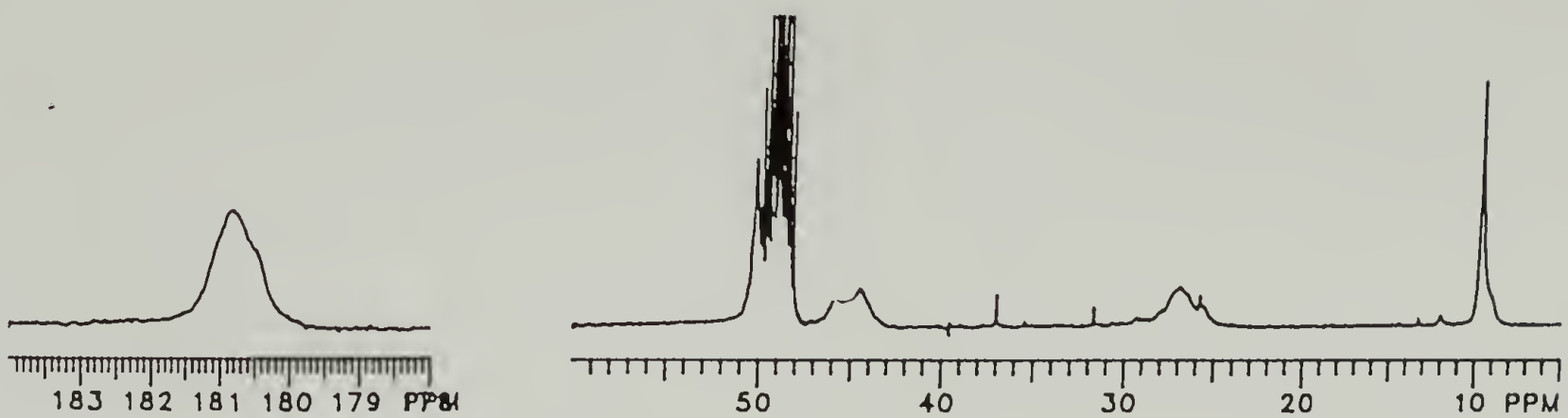
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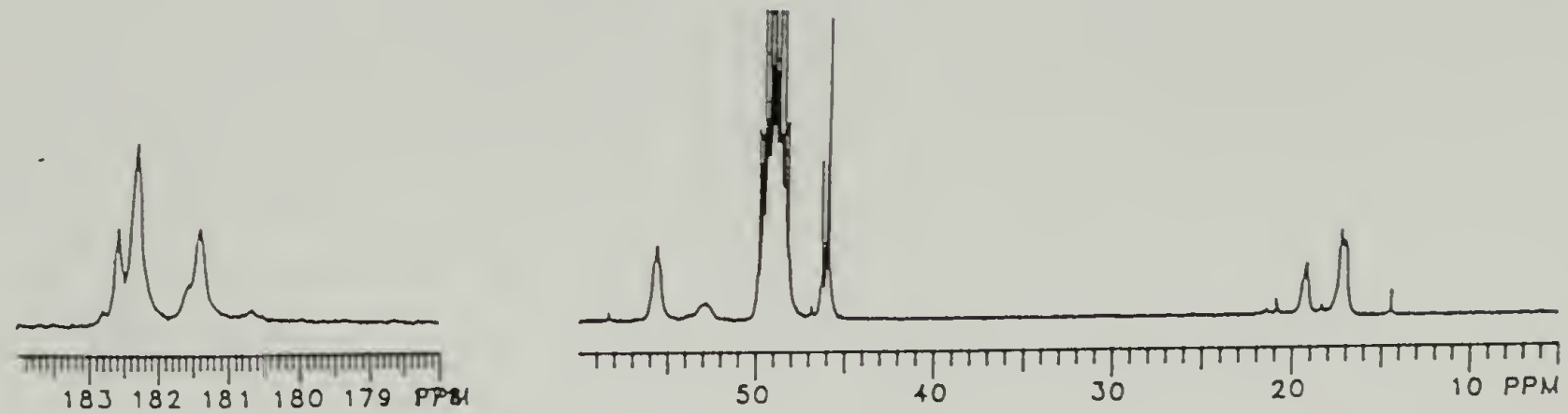
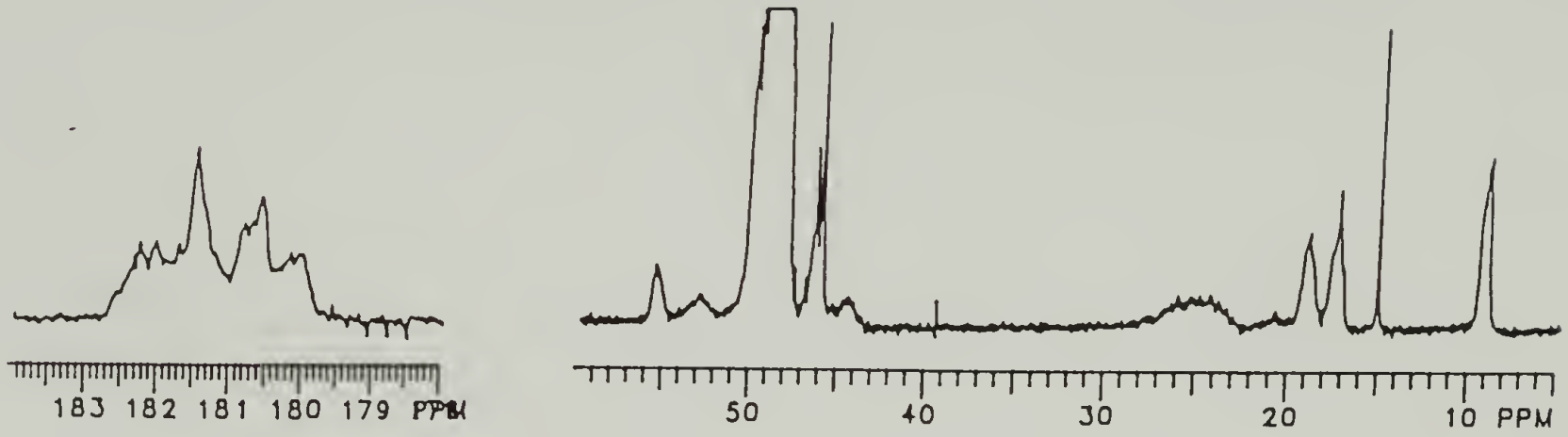

183 182 181 180 179 PPM
50
40
30
20
10 PPM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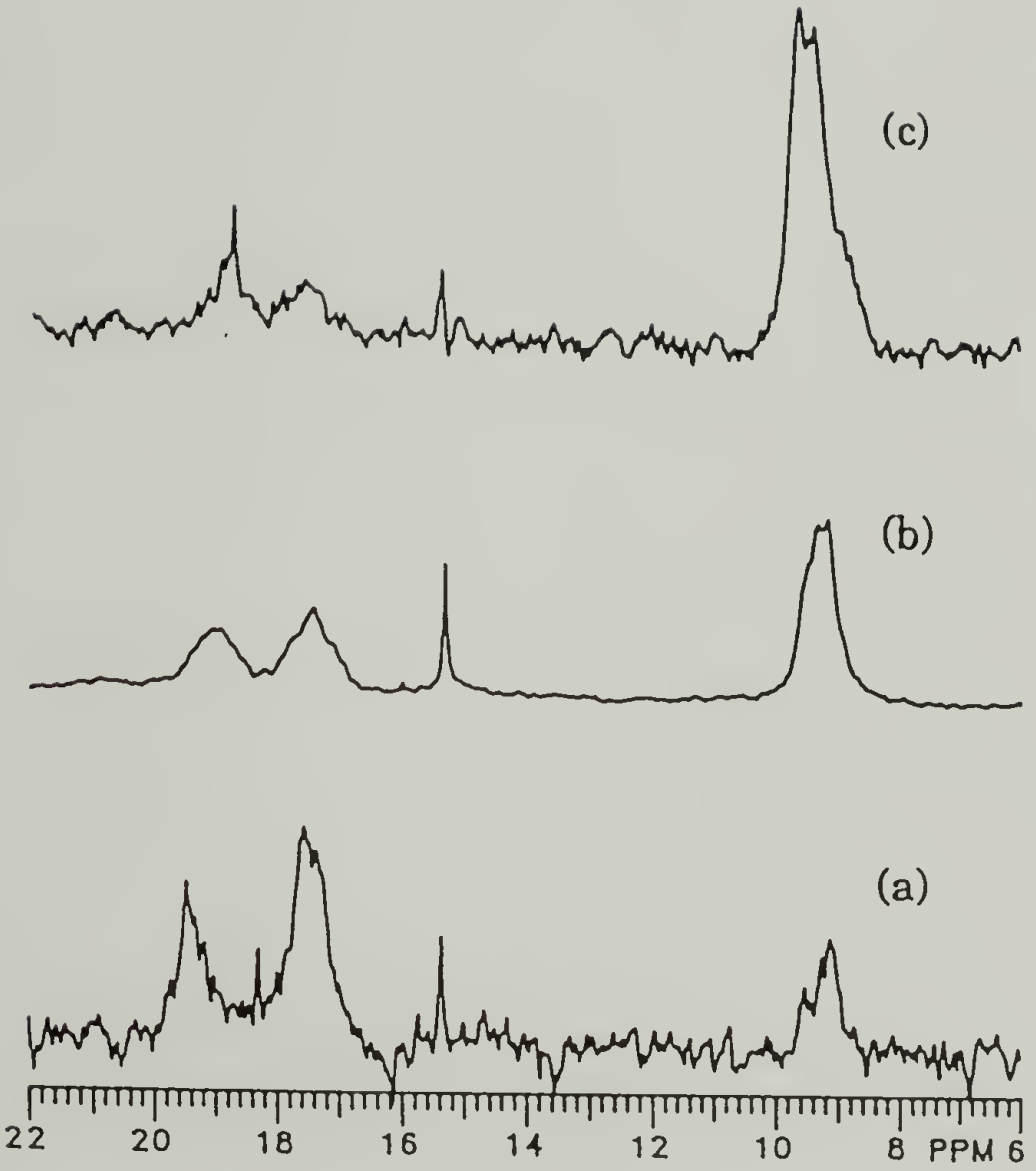
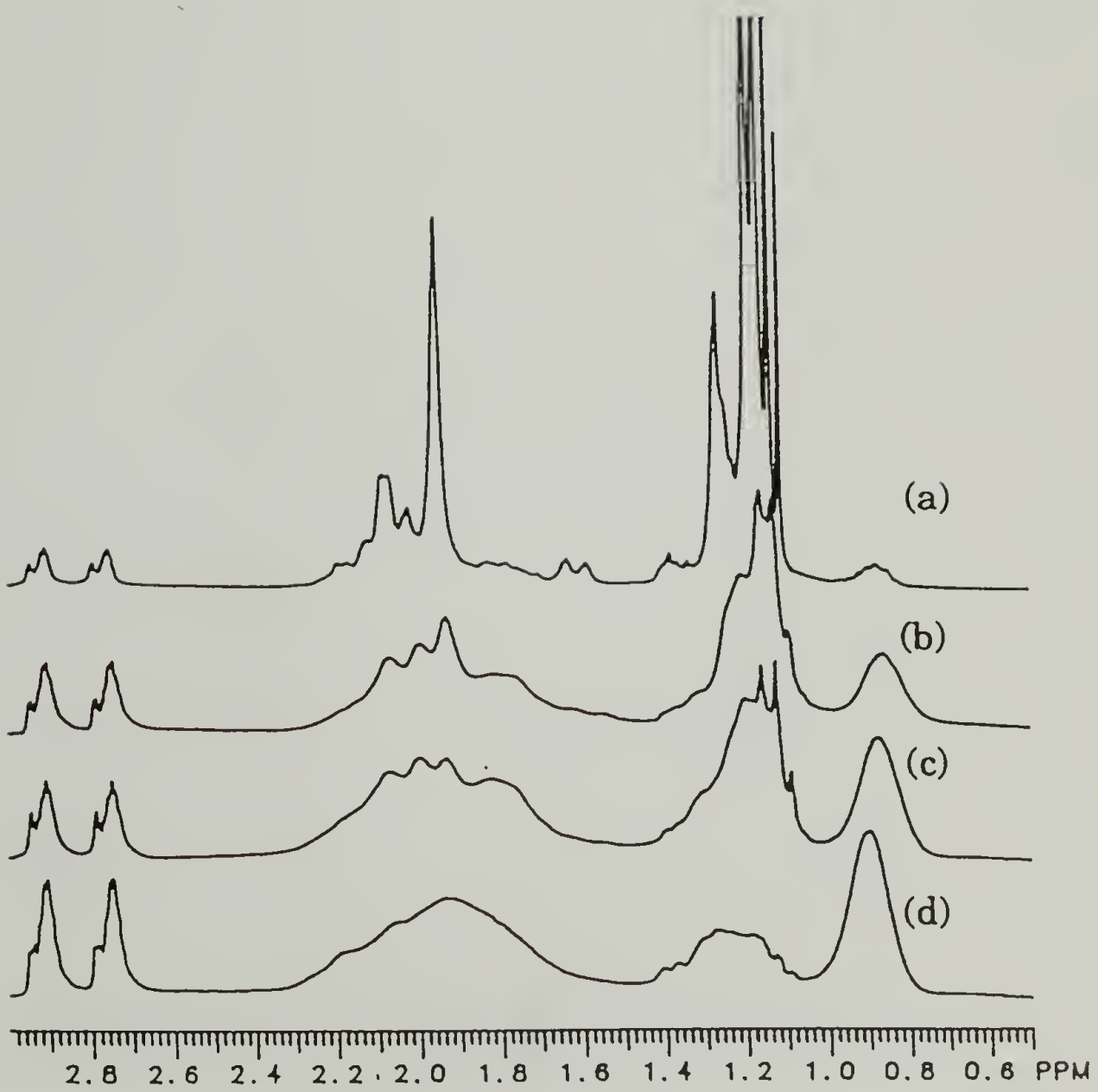
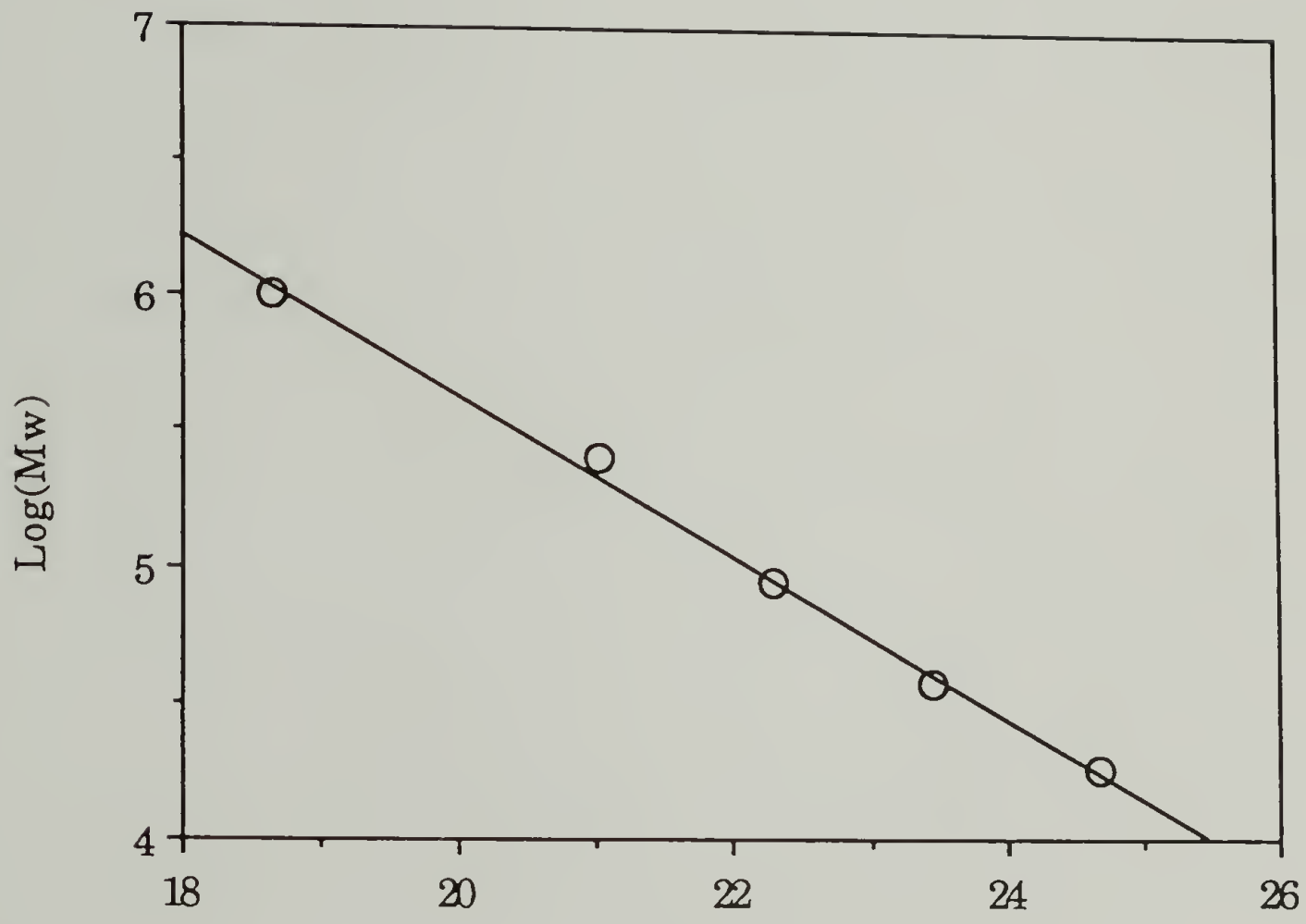
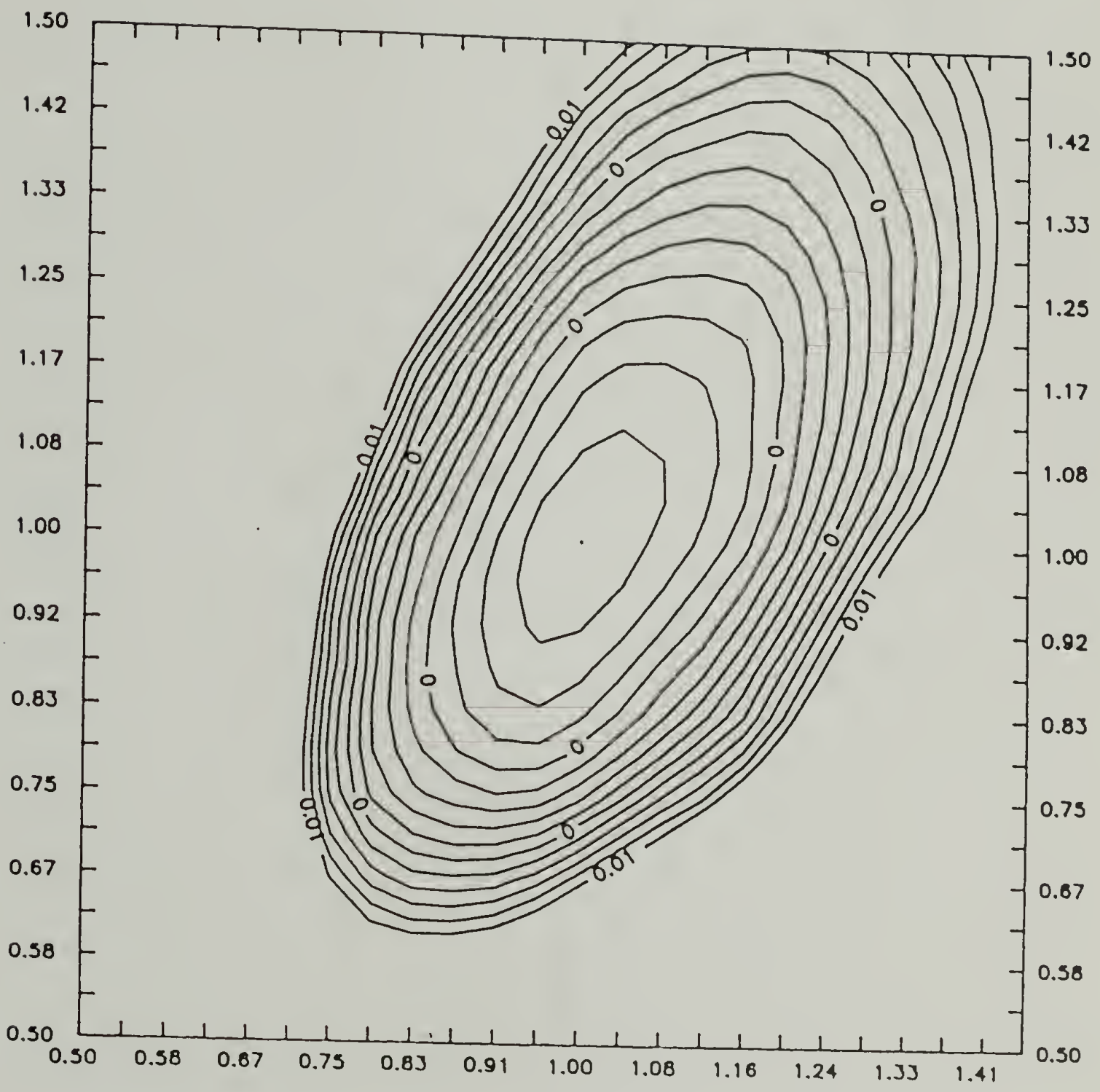
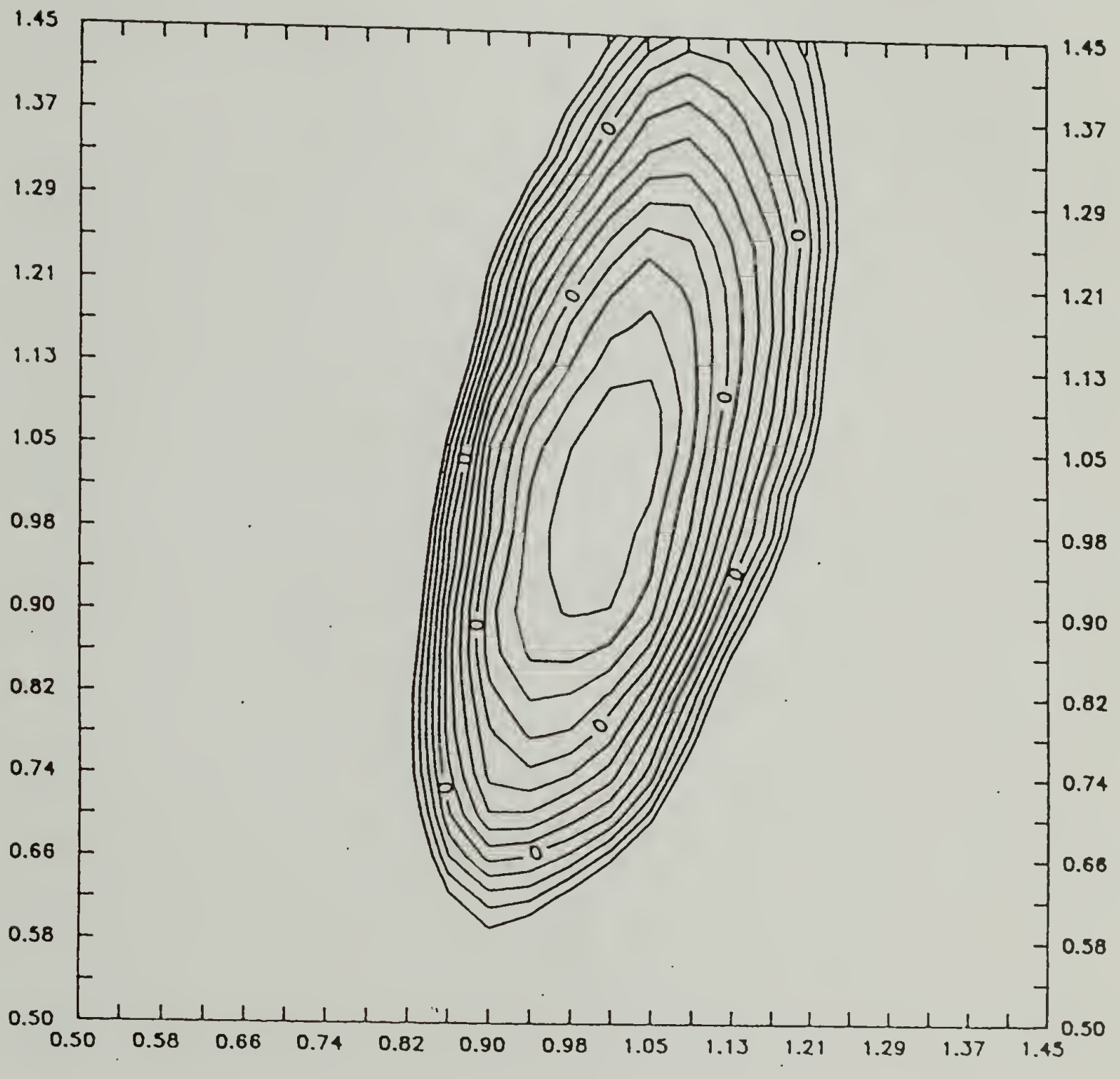
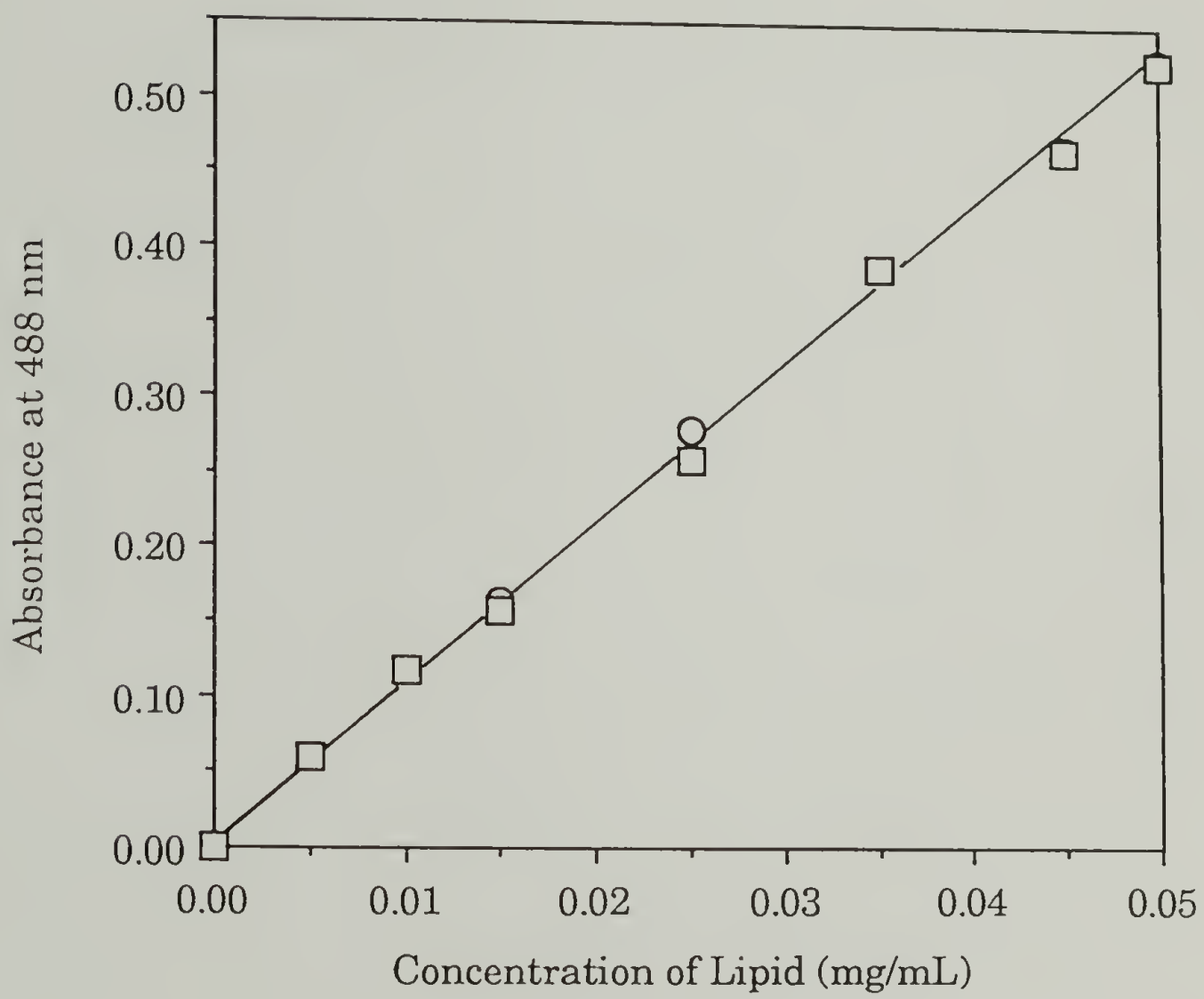

(c)
(b)
(a)
22
20
18
16
14
12
10
8
PPM
6

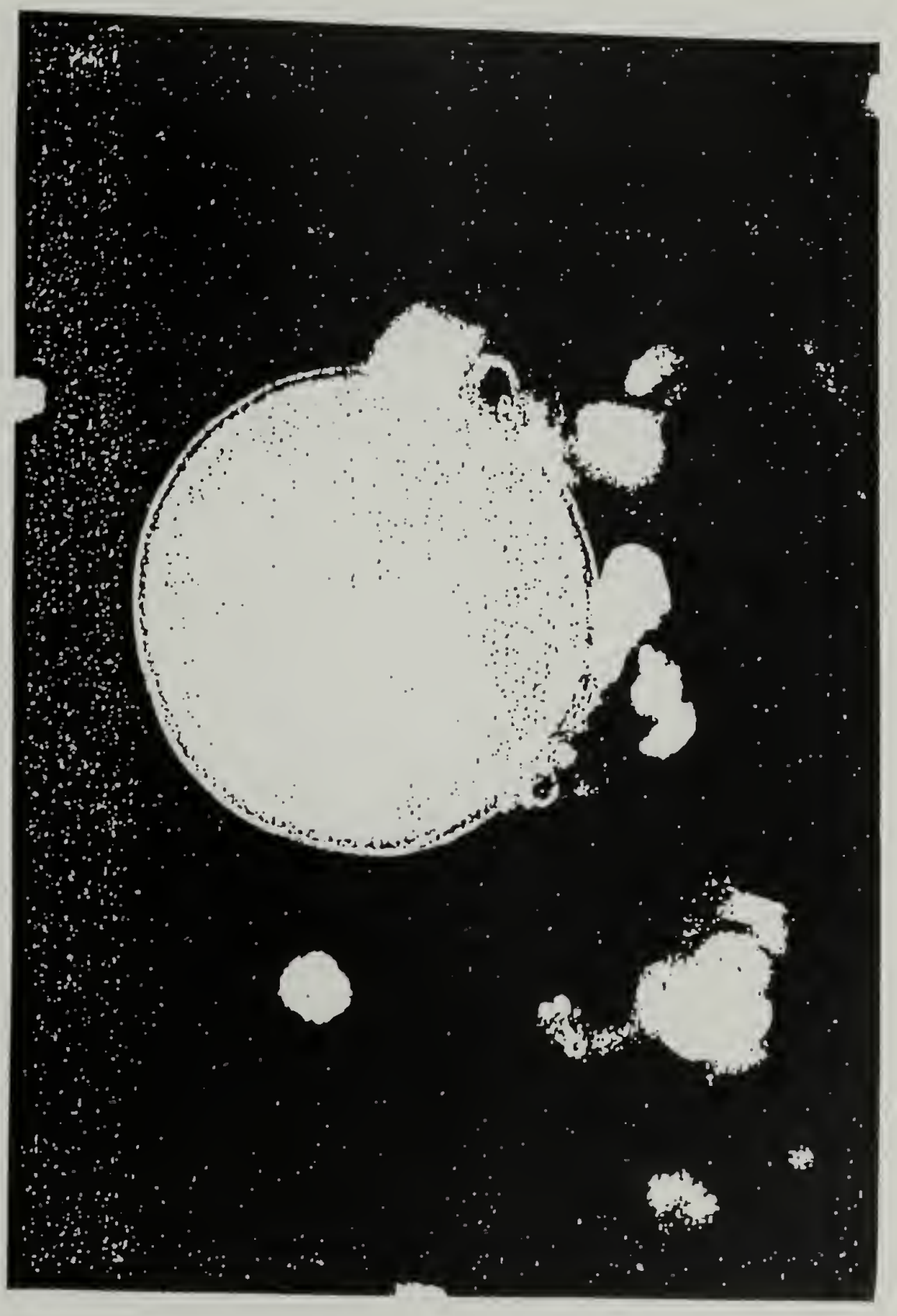

(a)
(b)
(c)
(d)
2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 PPM


7
6
5
4
Log(Mw)
18
20
22
24
26
Retention Volume


1.50
1.42
1.33
1.25
1.17
1.08
1.00
0.92
0.83
0.75
0.67
0.58
0.50
0.01
0
0.50
0.58
0.67
0.75
0.83
0.91
1.00
1.08
1.16
1.24
1.33
1.41


1.45
1.37
1.29
1.21
1.13
1.05
0.98
0.90
0.82
0.74
0.66
0.58
0.50
0.50 0.58 0.66 0.74 0.82 0.90 0.98 1.05 1.13 1.21 1.29 1.37 1.45


Absorbance at 488 nm
0.50
0.40
0.30
0.20
0.10
0.00
0.00
0.01
0.02
0.03
0.04
0.05
Concentration of Lipid (mg/mL)



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